Whooping cough vaccines: production of virulent *B. pertussis*

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Calling on all dreamers,
don't you ever wake up

CONTENTS

CHAPTER 1

General Introduction

1. Introduction

Whooping cough is a highly contagious disease that infects the human respiratory tract. The disease derives its name from the characteristic severe hacking cough followed by an intake of breath that sounds like 'whoop'. The coughing fits in young babies and children can lead to vomiting, turning blue due to insufficient breathing (cyanosis) and even temporary cessation of breathing altogether (apnoea). Due to complications of these symptoms mortality in unvaccinated individuals was and still is not uncommon. *Bordetella pertussis,* which is one of the two bacteria responsible for the disease, was already identified in 1906 by J. Bordet (Figure 1). In 1938, Eldering and Kendrick discovered the second bacterium that could cause whooping cough, i.e. *Bordetella parapertussis*. Since *B. parapertussis* generally causes less severe whooping cough, all vaccines are still based on *B. pertussis* only. Before mass vaccination, whooping cough was a major cause of child morbidity and mortality. The introduction of *B. pertussis* vaccines in the 1940s dramatically reduced the health burden caused by whooping cough and virtually eliminated infant mortality caused by *B. pertussis*.

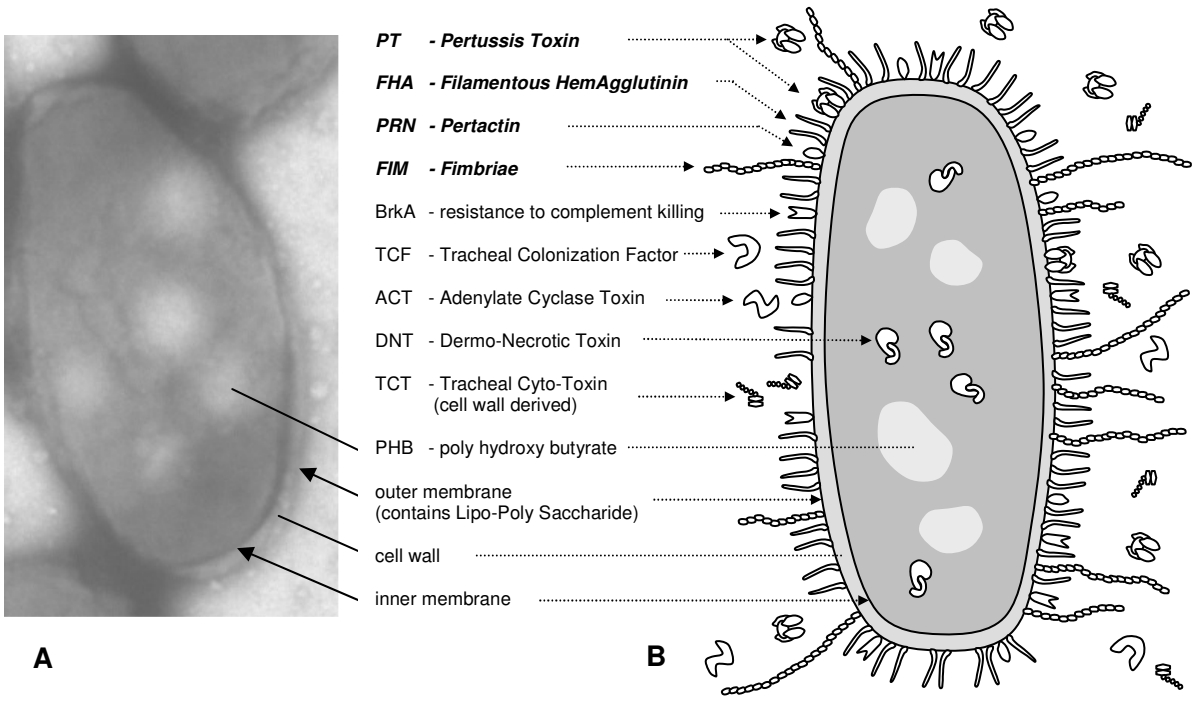

Figure 1. A: Electron microscopic picture of *B. pertussis*. B: schematic representation of *B. pertussis*, including the antigens and structures that play an important role in the ability of *B. pertussis* to cause whooping cough. The antigens that are components of acellular whooping cough vaccines are indicated in bold italics.

The first whooping cough vaccines were prepared by Madsen in 1933. The production method was straightforward: bacterial cells were grown, separated from the supernatant, resuspended in saline and inactivated using heat and/or chemicals. Essentially, this production process is still in use today by a number of large pharmaceutical companies such as GSK, Novartis and the Serum Institute of India, that supply the developing world with whole cell vaccines. Although the resulting, so called, whole cell vaccines were highly effective, they had and still have a number of inadequacies. Establishing the quality of the vaccine was and is a major problem of the vaccine, because an accurate animal model that corresponds with protection in humans is not available. Also, due to the lipo-poly saccharide (LPS) in the outer membrane of *B. pertussis* (Figure 1), a number of mild adverse events such as fever occur fairly frequently. The far more rare, yet more serious adverse events such as convulsions and collapse as well as unfounded allegations that whole cell vaccines cause neurological damage, gradually led to a demand for a whooping cough vaccine with less adverse events. The resulting acellular vaccines, i.e. vaccines that only consist of one or more *B. pertussis* antigens, were developed during the 80's and 90's. Though generally less effective in field trials than a good cellular vaccine, the adverse events were also less. Therefore, in most countries in the western world, after being used for half a century, cellular vaccines were replaced by acellular vaccines at the turn of the millennium.

Unfortunately, this brief overview of the history of whooping cough vaccines does not have a happy ending. For the last 2 decades, the incidence and the severity of the disease has increased, also in fully vaccinated individuals. Although most populations around the world are highly vaccinated, outbreaks of whooping cough are fairly common in the Western world as well as in the developing world. In 1999, the global number of whooping cough cases was estimated to be about 48.5 million, with 300.000 deaths (Crowcroft, Stein, Duclos & Birmingham, 2003). Also, *B. parapertussis* rather than *B. pertussis* is isolated more frequently from seriously ill whooping cough patients. In fact, in a number of countries *B. parapertussis* is responsible for up to 30% of the whooping cough cases (review Watanabe, 2004). Therefore, there is a definite need for an improved whooping cough vaccine.

Both the Netherlands Vaccine Institute (NVI), as well as the National Institute for Public Health and the Environment (RIVM) have played and still play an important role in the field of whooping cough vaccines ever since the introduction of vaccines in the Netherlands. The NVI is the result of a merger between the Foundation for the Advancement of Public Health and Environmental Protection (SVM) and the Sector Vaccines of the RIVM in 2003. The unique combination of vaccine production, public health surveillance as well as fundamental scientific research on one site in Bilthoven has resulted in a number of significant

contributions to the field of whooping cough during the last century. Since the work in this thesis was carried out at the RIVM/NVI, it is only fitting to describe the history of whooping cough vaccine development from the perspective of the RIVM/NVI. Due to the fact that this thesis primarily deals with vaccine development and vaccine production, the many significant contributions of the RIVM surveillance departments are not discussed in this overview. This overview highlights some of the unique problems related to whooping cough vaccines, parts of which are also addressed by this thesis as elaborated upon in the last paragraphs of this Chapter.

1.1 Vaccine development and production

Since the entire bacterial cell functions as a vaccine, the composition of the cell has a direct impact on the quality of the vaccine. In turn, the composition of the cell is a direct consequence of the conditions under which it is cultivated. Unfortunately, most of the media on which *B. pertussis* is grown are composed of complex raw materials such as yeast extract and hydrolyzed milk proteins. Since these materials have an inherent batch to batch variation, this influences the quality of the vaccine as well. Therefore, ever since the start of the development of whooping cough vaccines, the cultivation of the organism itself has been an important subject of research and development. After the discovery by Bordet (1906), a number of authors have worked on improving the cultivation media for *B. pertussis*. In the following decade at the RIVM, H.H. Cohen was responsible for the development and implementation of the whooping cough vaccine at the RIVM. After the introduction of the vaccine in the Netherlands in 1956, infant death (mortality) due to whooping cough virtually disappeared, while the infrequent cases of whooping cough in vaccinated individuals generally resulted in mild symptoms only.

At the RIVM and with other producers, the first whooping cough vaccines were made in glass, flat-bottomed Roux bottles. Typically these bottles contained less than a litre medium, and the bottles were left standing in a temperature controlled incubator without any form of agitation. Although this production method proved to yield potent vaccines, the method was not suitable to produce vaccines at large scale. Also, the number of manipulations required was prone to cause infections. Typically, the temperature was the only controlled parameter in the Roux bottles. Other important culture parameters such as the dissolved oxygen level or pH were neither measured nor controlled. In order to implement more uniform culture conditions and a more industrial approach to vaccine production in general, van Hemert and co-workers (Van Hemert, 1971, thesis) developed an, at the time, state of the art line of bioreactors, processing vessels and other equipment specifically designed for vaccine production (Figure 2). This

equipment allowed scaling up the production to 350 L, which was more than sufficient to supply the Netherlands and its colonies at the time with vaccines against diphtheria, tetanus and pertussis.

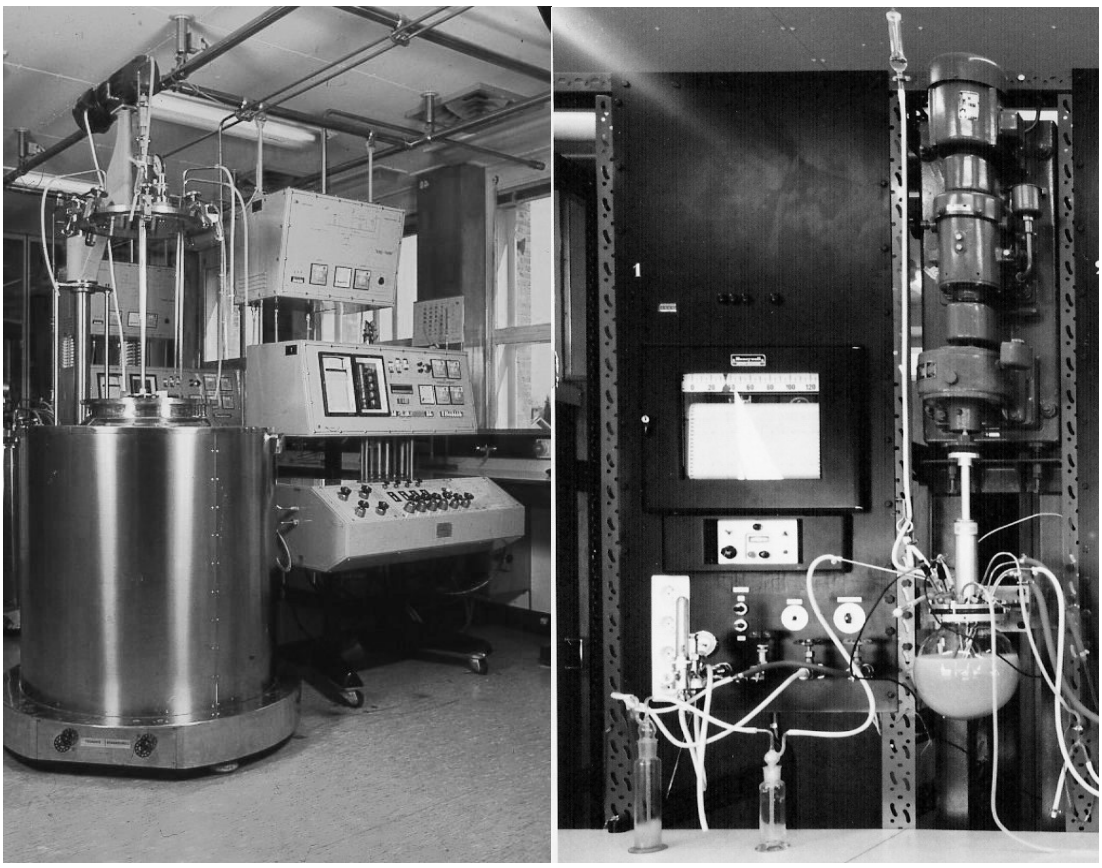

Figure 2. Pictures of some of the equipment specifically designed for vaccine production. Left: a 350 L bioreactor including the 'Novo-Paljas' measurement and control unit. Right: the first 'Bilthoven measurement and control unit', including a glass bioreactor (from: NVI-archive).

The use of a bioreactor allowed far better control of the culture conditions, as well as the study of the bacterium *B. pertussis* itself. During this period the B2 production medium was developed, which was used with only minor modifications for over 40 years (see Chapter 4), up until the production of cellular pertussis ceased in 2005. The studies by Van Hemert in his thesis (1971) yielded many interesting data about the bacterium and the use of continuous culture in vaccine production. Also, various harvesting techniques were tested such as the use of mild acid precipitation rather than centrifugation to separate the cell suspension from the supernatant (Van Hemert, 1969). Although technically this separation method worked very reliably, the resulting vaccine appeared to be somewhat lower in potency than the vaccine prepared by centrifugation. Despite these innovative approaches to vaccine production, the potency of the pertussis vaccine was not significantly improved over this period.

A different approach to decreasing the adverse events of a whole cell vaccine was taken by H.J. Hamstra (1995). Rather than using the whole cell as a vaccine, Hamstra extracted outer membrane vesicles from *B. pertussis* cultures. These vesicles were immunogenic in mouse models, and these preparations passed the Kendrick potency test, which are also applied to whole cell vaccines. By modifying the extraction method of the vesicles, the amount of LPS was reduced 100-fold. Furthermore, a relation between the content of a 92 kDa outer membrane protein and the Kendrick test seemed to indicate that the 92 kDa outer membrane protein could aid in the protection against whooping cough. Though this vesicle vaccine appeared to be quite promising, this vaccine formulation was not pursued further.

The introduction of computerized measurement and control systems at the RIVM by E.C. Beuvery in the 90's enabled new possibilities for the control of *B. pertussis* cultures. Aside from the culture information in the form of pH, temperature and dissolved oxygen, a computerized control system can also for example register the amount of oxygen needed to maintain the appropriate dissolved oxygen level. This type of information is a reflection of the amount and type of metabolic activity of the cells in the bioreactor, and can be used to determine at what point in time all substrates are depleted. For example, at production scale, in Chapter 4, a clear relationship between the consumption of substrates and the control of the dissolved oxygen was established, in order to predict the appropriate time to harvest. Using the chemically defined THIJS medium (described in Chapter 3 of this thesis) as well as the feed-medium defined in Chapter 5 of this thesis, Neeleman (2001, Thesis, Chapter 8) took this approach one step further and programmed a feeding algorithm in the software of the measurement and control system that was capable of controlling the growth rate of *B. pertussis* for a number of hours.

Like all biotechnological products, a whole cell vaccine is defined by the production process and by the off line analytical assays used to measure certain product related parameters. The application of a Near Infra Red (NIR) probe has allowed a very detailed insight into the events that take place inside the bioreactor. At the NVI, Streefland (2007) used the combination of the NIR probe and DNA arrays to link the events taking place in the bioreactor with the events taking place at the cellular level. This approach enabled the identification of genes that correspond with vaccine quality, thereby allowing a far more quantitative description of the quality of the whole cell vaccine than any animal model can provide. Although the cellular vaccine is no longer produced at the NVI, the knowledge gained about this production process is applicable in other production processes of biological products.

1.2 Development of animal models to study adverse events of whole cell vaccines

The main disadvantage of cellular whooping cough vaccines is that these have a fair amount of frequent adverse events such as fever, pain around the injection site, etc.. Many of these adverse events are caused by LPS, a component of the outer cell membrane of *B. pertussis* cells (Figure 1). While there are a number of ways to measure the LPS content of whole cell vaccines, the most predominant side effect of whole cell vaccination, fever, cannot be directly linked to *in vitro* methods. To predict fever in humans, a rabbit model was developed in 2000. In this model the rabbit temperature was measured continuously using a remote sensor, allowing the animals to move around freely. Initial results showed a good correlation between the onset of fever in rabbits and the typical start of adverse events in humans such as fever and crying, that start approximately 3 hours after vaccination. In a more sophisticated setting, Verwer (in press) was able to show clear differences in the temperature profile of rabbits, that were vaccinated with a full dose and half a dose of whole cell vaccine as compared to the temperature profile of rabbits that were vaccinated with saline. An interesting observation in this study was that the housing conditions and habituation to human handling, have a significant influence on the degree of fever experienced.

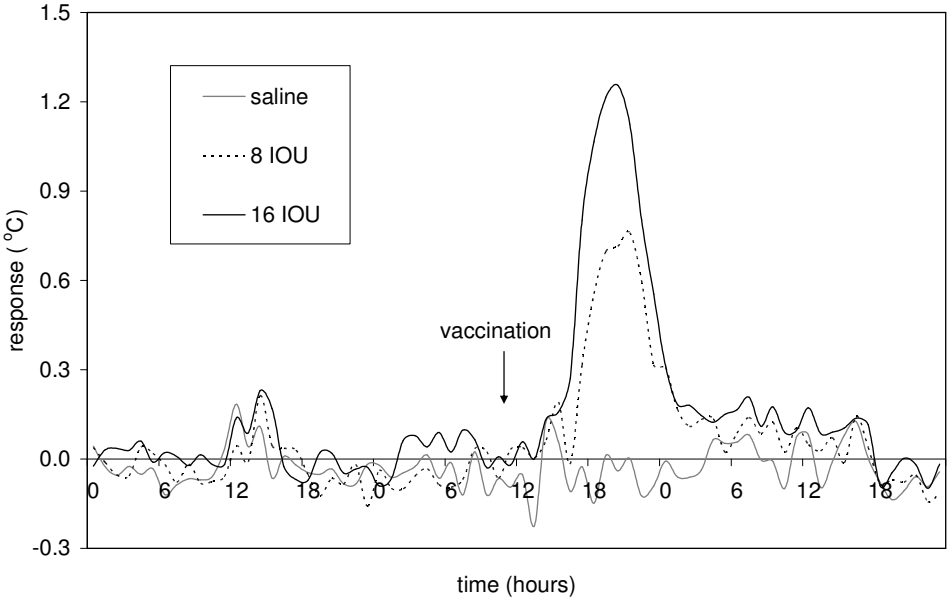

Figure 3. Differences in body temperatures of rabbits that were vaccinated with saline (grey base line), half a human dose (dotted line) and a full human dose (solid line). The time of vaccination is indicated with an arrow (from: Verwer, in press).

Whole cell vaccines cause rare adverse events such as convulsions and collapse. The RIVM has aimed to define the cause of these rare adverse events. For example, De Wildt (1983) found that whole cell vaccination in rats caused the basal blood pressure and other responses of the cardiovascular system to be significantly lower than those of the control rats. This effect lasted up until 4 days

after vaccination. Kreeftenberg (1984, 1985) investigated the effect of whole cell vaccination on the serum glucose content after vaccination in mice and found that whole cell vaccination resulted in low glucose levels for periods up to a week, mainly due to LPS and pertussis toxin (PT). These observations could explain why some of these rare adverse events occur, although it is inherently difficult to directly link a rare side effect to animal models.

1.3 Development of *in vitro* tools and animal models related to potency

The mouse model developed by Kendrick in 1947 predicts the ability of a cellular vaccine to protect against whooping cough in man, i.e. the potency of the vaccine. The limited accuracy of this Kendrick test is not surprising given the nature of the model. Mice are vaccinated in the abdomen and, after 2 weeks, infected intracerebrally with living *B. pertussis* cells. Again two weeks later, the number of surviving mice of the test vaccine is compared to the number of surviving mice of a reference preparation. Typically, the outcome of the test has a margin of 40 to 250% of the value of the test, i.e. the 95% confidence limits are quite wide. Aside from causing a great deal of animal suffering, the inaccuracy of this mouse model has hampered optimisation of the vaccine, since moderate differences in the quality of the vaccine cannot be measured accurately enough. Despite the limitations of this test, even today this is the mandatory model by which the potency of cellular vaccines is tested. Also the vaccines described in Chapter 4 were tested extensively with the Kendrick test.

Over the last two decades the RIVM/NVI developed several animal models that enabled a more accurate estimation of the potency as well as of the toxicity of whole cell vaccines. A most important contribution was the development of a Pertussis Serological Potency Test (PSPT) in mice (van der Ark, 1994, 1996), in which vaccinated mice were not challenged intracerebrally, but antibodies were measured in the serum. The total antibody concentration against *B. pertussis* correlated quite well with the survival of mice in the Kendrick test (Figure 4). This PSPT was later assessed in a collaborative study (van der Ark, 2000). Not only is the serological model more accurate than the Kendrick test, i.e. the 95% confidence limits are significantly smaller, the test also causes far less animal suffering, since the vaccinated mice are not infected intracerebrally with *B. pertussis*. While this test is not in use as a mandatory test to estimate the potency of a whooping cough vaccine, a modified version of this test has proven invaluable for generating reliable data regarding the potency of experimental vaccines. Finally, van Straaten (2002) managed to combine the Mouse Weight Gain, the Leucocytosis Promotion, and the PSPT in a single mouse model, i.e. toxicity, pertussis toxin activity as well as potency can be assessed in the same

test, with minimal animal suffering. Most of these models have been used in order to test the experimental vaccines described in Chapter 4.

It is logical to assume that the amount of the various antigens in the whole cell vaccine corresponds with its capability to protect against whooping cough. Therefore, it is necessary to measure the quantity of these antigens in a cellular vaccine. While Ibsen (1993) analysed whole cell extracts for the content of a number of important *B. pertussis* antigens, at the RIVM J. Westdijk (1997) developed an ELISA that is capable of directly measuring the cell associated antigens in a whole cell vaccine. This allowed the detailed batch to batch comparison of whole cell vaccines, as well as the composition of cells at the various growth stages of the cultivation. For this thesis, the quantification of antigens using this method was vital during the initial medium optimisation for antigen expression described in Chapter 3.

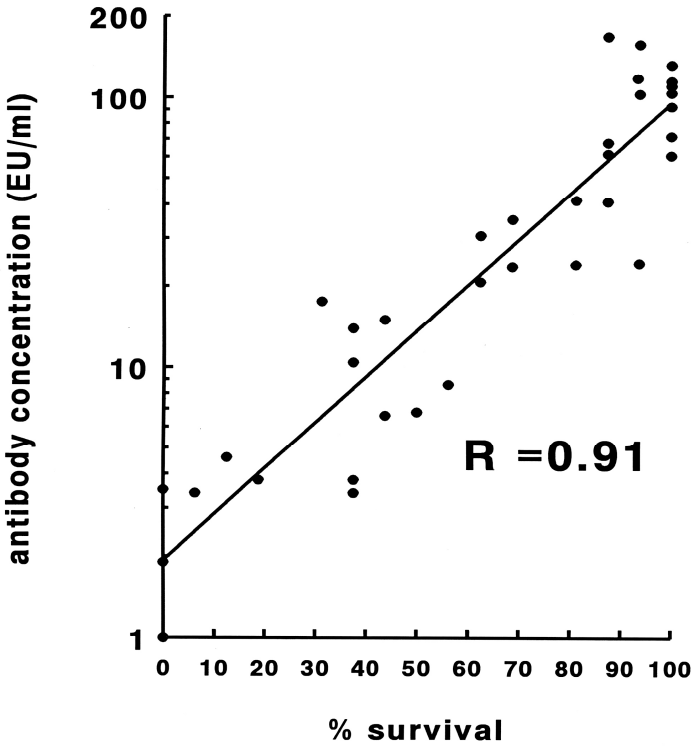

Figure 4. Comparison between the Kendrick and the PSPT test showing the correlation between the antibody titers in the blood of vaccinated mice and the percentage survival in mice that were challenged intracerebrally after vaccination (from: van der Ark, 1994).

1.4 Acellular vaccines and antigenic variation

Since the whole cell vaccines caused a number of adverse events, scientists have tried to identify which single antigen of *B. pertussis* could replace the whole cell vaccine. As part of the Laboratory for Bacterial Vaccines at the RIVM, J. Nagel undertook a considerable effort in separating and identifying this hypothetical 'protective antigen' of *B. pertussis* (1967). Although he did not succeed in finding this hypothetical antigen, his work on *B. pertussis* extracts did yield a number of significant discoveries with regard to some of the toxic antigens produced by *B. pertussis*, as well as the immunological properties of *B. pertussis* extracts (Nagel 1971, Thesis).

In Japan the public acceptance of the adverse events of whole cell vaccines decreased dramatically during the 70's and 80's, which led to the development of vaccines that consisted of partly purified extracts of *B. pertussis* cultures. Since these extracts contained much less LPS, the adverse events were less as compared to the whole cell vaccine. A couple of these products are still in use today. In the 90's a number of acellular vaccines were tested in large clinical trials that consisted of fully purified *B. pertussis* antigens that were either genetically or chemically inactivated. Since these vaccines provided adequate protection against whooping cough and have considerably less adverse events than most whole cell vaccines, most countries switched to acellular vaccines around the year 2000.

Whole cell and acellular vaccines alike are derived from *B. pertussis* strains that were isolated in the 50's. Research at the RIVM by F. Mooi (1998) has shown that *B. pertussis* strains isolated the last 3 decades from whooping cough patients differ at the genetic level for a number of antigens, notably for PT and pertactin (PRN). Figure 5 shows that during the 70's, the isolated strains from patients that contained PRN1 gradually disappeared, while in this millennium no strain with PRN1 is isolated anymore. Both PT and PRN are included in most acellular vaccines. This suggests that the wide spread use of vaccines based on the original strains has lead to mutations in the genes of the strains that circulate in humans. This hypothesis is consistent with the re-emergence of whooping cough in highly vaccinated populations, (Mooi, 2001).

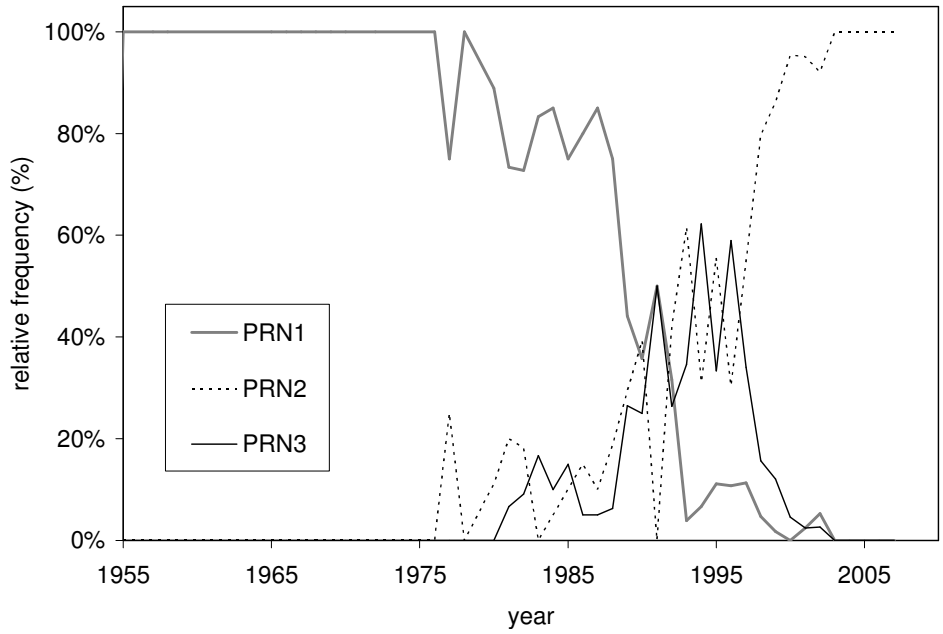

Figure 5. Temporal trends in the relative frequency of PRN variants in the Dutch *B. pertussis* population. The percentage of strains harboring distinct PRN variants was determined in Dutch strains collected between 1955 and 2007 for each period indicated on the *x* axis. Strains collected between 1955 and 1975 contained the PRN1 variant only, which is the variant present in the Dutch vaccine strains (from: Mooi, 2007).

1.5 Contents of this thesis

As mentioned before, the ability to cultivate *B. pertussis* has been a subject of study ever since the start of whooping cough vaccine development. This thesis about whooping cough vaccines is no exception: in 4 of the 7 Chapters a number of essential questions regarding *B. pertussis'* metabolism are answered. *B. pertussis* cannot metabolize glucose, and has mostly been cultivated on media with an abundance of amino acids, which results in low growth rates, low biomass yields in terms of gram biomass per mole of carbon consumed and low absolute yields in terms of dry weight biomass per liter, typically less than 1.5 g/L. In order to cultivate *B. pertussis* reproducibly, a thorough understanding of the metabolic capabilities of the organism is vital. Therefore, in the 2nd Chapter of this thesis, the basic metabolism of *B. pertussis* was mapped by testing whether or not a number of substrates can be metabolized by *B. pertussis*. Using NMR to measure all metabolites in the supernatant and cytoplasmic extracts, a number of metabolic capabilities were revealed that were previously unknown. While conventional media use glutamate as the dominant substrate, this thesis showed that a combination of glutamate and lactate yielded faster growing cultures, without the formation of excess ammonium, which is toxic to the cells at high concentrations. Also, it was shown that *B. pertussis* can produce intracellular

poly-hydroxy butyrate (PHB) in significant amounts (Figure 1), especially in complex media. This product does not add to the quality of a whole cell vaccine, and should therefore be avoided. By choosing the appropriate medium composition and conditions, PHB production can be avoided, which has a positive effect on the yield of bacterial mass per mole carbon consumed. Typically literature reports yields of 6 to 7 gram biomass per C-mole (Licari, 1991, Frohlich, 1995), whereas we found yields of 8 to 9 gram biomass per C-mole. By applying the metabolic information gathered, a chemically defined medium was formulated that allowed growth of *B. pertussis* without waste metabolism, at high yields (Thalen, 1999).

Since it is reasonable to assume that the amount of antigens expressed on the surface of the *B. pertussis* cells is related to the potency of the whole cell vaccine, the medium developed in Chapter 2 was optimised in order to increase the expression of PT. PT expression is representative for all antigens expressed by *B. pertussis*. Several factors are known to influence the expression of *B. pertussis* virulence factors, such as the Na^+ concentration, the iron concentration and the temperature. While mostly these parameters were used to examine expression versus no expression, this was the first time that these parameters were tested over a broad range. Next to PT expression, also the association of PT to the cells was measured, since PT and other antigens excreted into the supernatant are typically lost during the processing of a cellular vaccine. After optimizing the medium for PT expression, the yield of PT per gram biomass reached a level of approximately 2.0 mg/g, which was mostly cell associated. Also, the activity of a heat sensitive protease produced by *B. pertussis* was monitored in order to select conditions that favoured high expression of antigens, while limiting the degradation of these antigens by the protease. The resulting Thalen-IJssel (THIJS) medium (Thalen, 2006) is suitable for optimal expression of *B. pertussis* antigens, largely associated to the cells, with very limited degradation of the antigens.

The THIJS medium was tested on production scale (350 - 1000 L) to verify the suitability of the medium for whole cell vaccine production, which is described in Chapter 4 (Thalen, 2008). In terms of dry weight, approximately 1 gram of biomass was produced per litre of medium, which approximately corresponds with 1200 doses of whole cell vaccine, which is somewhat lower than the yield on a conventional complex medium. Next to implementing the THIJS medium, a number of steps of the production process of the conventional cellular vaccine was investigated in order to minimize batch to batch variability. The resulting vaccine suspensions had a potency that was 2 to 3 fold higher than the conventional product produced with the same equipment. A two year real time stability study showed that the products resulting from this production process retained the high potency compared to the conventional product.

The acellular vaccines described earlier, are more expensive to produce than cellular vaccines due to the extensive purification process and the fact that 5 to 25 times as many *B. pertussis* cells are needed to produce one dose of acellular vaccine as compared to one dose of cellular vaccine. For example, one litre of culture broth of THIJS medium yields approximately 1200 doses of cellular vaccine. Given a fairly high yield of PT of 2 mg/L on THIJS medium, and an often used PT content per dose of 25 µg per dose acellular vaccine, this would result in only 80 doses per litre of culture broth. This is a 15-fold lower yield of doses per litre fermentation volume as compared

to the yield of a cellular vaccine, without taking the losses during purification into account. One way to limit the cost of production is to produce more biomass per cultivation, which can be achieved by changing from a batch process to a fed-batch process. Therefore, in the 5th Chapter of this thesis a feed medium and feeding strategy were developed in order to maximize biomass production, limit waste metabolism and retain the high production of PT. The resulting fed-batch process produced 7 times the amount of cells as compared to the batch process (Thalen, 2006). As such, the THIJS medium and feed medium can be used to generate antigen containing biomass in a more cost effective manner as compared to conventional batch media currently in use.

A view on future whooping cough vaccines is expressed in the last Chapter of this thesis, extrapolating the history of cellular vaccines to acellular vaccines, while taking a number of disturbing recent developments with regard to the epidemiology of whooping cough into account. From this analysis it is clear that currently registered vaccines need to be improved in order to: (1) protect infants from an earlier age onwards, (2) vaccinate adolescents and adults (3) reduce the adverse events of acellular vaccines, and (4) increase the duration of protection of the vaccine by matching the vaccine strains with the circulating strains. In order to fulfil these requirements in a timely and cost effective manner, an oral or nasal vaccine could be used rather than an injectable vaccine. Although at RIVM/NVI there is no experience with such whooping cough vaccines, literature in this respect looks quite promising. An oral or nasal vaccine type fulfils all 4 requirements, (1) an oral vaccine can be administered in the first week after birth rather than 2 months after birth, (2) adolescents and adults can be vaccinated repeatedly, (3) adverse events are absent, and last but not least, (4) by incorporating the circulating antigens the duration of protection may increase.

B. pertussis was discovered more than a century ago, and 3 generations of vaccines have been developed. Nevertheless, whooping cough vaccine development is still badly needed and exciting area of research and development.

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CHAPTER 2

Rational medium design for *Bordetella pertussis*: basic metabolism

Summary

In current *B. pertussis* media ammonium accumulates because of an imbalance in the nitrogen:carbon ratio of the substrates used, which is one of the factors limiting cell density in fed-batch cultures. The aim of this study was to map *B. pertussis'* catabolic and anabolic capabilities, in order to design a medium that avoids ammonium accumulation, while substrates are metabolised completely. Besides the known dysfunctional glycolysis, *B. pertussis* also possessed a partially dysfunctional citric-acid cycle. Although ammonium accumulation was avoided by adding various carbon sources to medium with glutamate, NMR showed excretion of acetate, acetoacetate and β-hydroxy-butyrate, thereby reducing the biomass yield. Acetoacetate and β-hydroxy-butyrate were also formed in Verwey, B2 and modified Stainer Scholte medium. Electron microscopy in combination with NMR showed that cells early on in these cultures contained poly-hydroxy-butyrate (PHB) globules, which disappeared later during the culture, coinciding with the appearance of β-hydroxy-butyrate and/or acetoacetate. No globules nor metabolite excretion was detected when lactate in combination with glutamate were used as substrates. Thus, metabolite excretion and ammonium accumulation were avoided, while the yield of 8.8 g per C-mol compared favourably with literature values, averaging 6.5 g per C-mol. Optimisation of this medium for PT production will be reported in a separate article.

Key words: *Bordetella pertussis*, medium design, ammonium accumulation, waste metabolism, NMR, poly-hydroxy-butyrate, PHB

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1. Introduction

Whooping cough is caused by the Gram-negative aerobic bacterium *Bordetella pertussis* or *Bordetella parapertussis*. The disease can effectively be controlled by immunisation with a vaccine consisting of inactivated *B. pertussis* cells. However, due to the adverse effects of vaccination with whole cells, a number of acellular vaccines were developed and tested in field trials (Edwards, 1995). Acellular vaccines contain *B. pertussis* virulence-factors, such as pertussis toxin (PT), filamentous hemagglutinin, and pertactin. The production of a whole-cell vaccine involves growing, concentrating and inactivating *B. pertussis* suspensions. Additionally, acellular vaccine production requires the isolation and purification of one or more virulence-factors from these suspensions. The concentration of for example PT in one dose acellular vaccine is higher than the concentration in one dose whole-cell vaccine as reported by Edwards *et al.* (1995) and Ibsen *et al.* (1993). Using their data, the number of doses acellular vaccine obtained per bioreactor volume can be estimated to be at best 5-fold lower than for whole-cell vaccines. The demand for large amounts of *B. pertussis* antigens to be used in acellular vaccines therefore calls for increased volumetric productivity of *B. pertussis* cultivations.

Since *B. pertussis* does not possess a functional glycolysis, the organism is usually cultivated on media with amino acids as carbon and nitrogen source. The molar nitrogen:carbon (N:C) ratio in the composition of *B. pertussis* is 1:6 (Licari *et al.*, 1991). Since a fraction of the substrate carbon is oxidised to carbon dioxide, the medium N:C consumption ratio for *B. pertussis* will be higher than 1:6. Glutamate and proline, the substrates used in the modified Stainer-Scholte (MSS) medium (Stainer and Scholte, 1971), both have an N:C ratio of 1:5, which results in ammonium accumulation during the culture, a characteristic of all current *B. pertussis* media.

The *B. pertussis* concentration in MSS medium is approximately 2 g l^{-1} dry weight (Andorn *et al.*, 1988). Compared to 40 g l^{-1} or more for *E. coli* cultivations (Yee and Blanch, 1992), it should be possible to increase bioreactor productivity by increasing the final cell density, while retaining expression of the major virulence-factors. Using glutamate as substrate in fed-batch cultures, Frohlich *et al.* (1995) achieved 8.8 g l^{-1} dry weight at relatively low PT production. They showed that a high concentration of most salts, including ammonium, decreases the growth rate and the final cell density. During their fed-batch cultivations, typically 120 mM ammonium was formed. When this amount was added to a bioreactor culture prior to inoculation, the growth rate declined more rapidly than the control culture, while only half the final density of the control culture was reached.

In order to balance the medium N:C ratio, a carbon source is needed that can be metabolised by *B. pertussis*. Studying the catabolic activities of washed *B. pertussis* suspensions, Jebb and Tomlinson (1951) found that asparagine, aspartate, serine, alanine, proline, and glutamate are oxidised, the latter 3 being favoured most by *B. pertussis*. Apparently amino acids degraded to pyruvate or α-ketoglutarate are rapidly oxidised. Jebb and Tomlinson (1951) deduced complete oxidation of proline, glutamate, serine and alanine, supposedly in the citric-acid cycle. Furthermore, they found that fumarate, malate and oxaloacetate were not oxidised, while α-ketoglutarate, succinate, pyruvate and lactate were. The latter compounds could in theory be used to decrease the nitrogen surplus as encountered in current *B. pertussis* media.

This study aims to design a medium for *B. pertussis* that avoids ammonium accumulation, while substrates are metabolised efficiently. Literature data were analysed and supplemented with results from shake-flask experiments to qualitatively map the main metabolic pathways of the bacterium, in order to identify substrates suitable to balance the N:C ratio of the MSS medium. In a forthcoming paper the medium designed here will be optimised for PT production.

2. Materials and Methods

Media. MSS medium was used with regard to salts and supplement, with varying amounts of glutamate and carbon sources as indicated in the text. Initial pH was always adjusted to 7.2 using 5 M NaOH. The osmolarity of the culture was adjusted to approximately the osmolarity of the MSS medium of 200 mOsmol by adding approximately 2.5 g l^{-1} extra NaCl, the exact amount depending on the substrates added. Basal medium composition in g l^{-1}: NaCl, 2.5; KH_2PO_4, 0.5; KCl, 0.5; $MgCl_2.6H_2O$, 0.1; TRIS, 1.525. Supplement composition in g l^{-1}: L-cystine, 4.0; $CaCl_2$ $2H_2O$, 2.6; glutathione, 10.0; $FeSO_4$ $7H_2O$,1.0; nicotinic acid, 0.4; L-ascorbic acid, 2.0 and 1 M HCl, 120 ml, using 10 ml l^{-1} basal medium. Both the basal medium and supplement were filter sterilised prior to use. In preliminary experiments, 90% L-lactate was used, containing ±30% lactate condensation products, not consumed by *B. pertussis*. Therefore for this study we used 40% L-lactate (Fluka cat.no. 69780), without lactides.

Strain, growth conditions. Strain 509 was used in all experiments, which is one of the two strains included in the DPTPolio vaccine applied in the Netherlands. Culture volume was always 200 ml in a 0.5 l flask, incubated a 35°C, on a 200 RPM rotary shaker. Verwey medium (Verwey, 1949) was inoculated with a lyophilised ampoule, incubated for 2 to 3 days until it reached 20±4 IOU, of which 10 ml was transferred to a second shake flask with Verwey medium. After incubation for 24 hours, 10% volume volume^{-1} glycerol (87% weight volume^{-1}) was added, then freezing the suspensions in aliquots of 10 ml at -70°C. The preculture medium containing glutamate and/or other carbon sources was inoculated with 10 ml of these -70°C working seed lots. The experimental cultures were inoculated with 2 ml of the preculture, i.e. a 1% volume volume^{-1} inoculum. Initial experiments were always confirmed in more elaborate cultivation experiments.

Biomass determinations. The optical density was measured using a Vitalab 10 (Vital Scientific, Dieren, the Netherlands), at 590 nm. One optical density unit corresponds to 20 International Opacity Units (IOU). Dry-weight determinations were done by centrifugation at 8000 g for 10 minutes using the complete culture volume after the optical density had become stationary or decreased. The cell pellets contained less than 0.5% water after 24 hours at 80°C, as determined in a Mitsubishi moisture analyser (Mitsubishi, Tokyo, Japan).

Metabolite determinations. Ammonium and α-ketoglutarate were determined with an enzymatic kit (Boehringer, Mannheim, Germany). Glutamate was determined using an HPLC method as described before (Dorresteijn *et al.* 1996). Lactate was determined with a YSI 2700 glucose/lactate analyser (Yellow Springs Instruments Co., Yellow Springs, USA).

Electron microscopy. Cells were washed in saline, adsorbed to glow-discharged carbon-stabilized formvar-coated grids, negatively stained using 2.0% ammonium molybdate (pH 5.1) or 2.0% potassium phosphotungstate (pH 6.0) and analyzed in a Philips TEM400 electron microscope (Philips, Eindhoven, the Netherlands) at an

operating voltage of 80 kV. Images were digitally stored and analyzed using analySIS 2.1 (Soft-Imaging Software, Munster, Germany). Images were printed after modifying their grey-value.

Isolation and analysis of poly-hydroxy-butyrate. Cells were collected by centrifugation at 8000 g for 10 minutes at relevant culture times. Cells were resuspended in 5 ml buffer pH 7.5, containing EDTA, 1 mM; TRIS 50 mM; $MgCl_2$, 5.4 mM, Lysozyme 0.3 mg ml^{-1}, Benzonase (Sigma, E8263), 65 U ml^{-1}. The suspension was extracted twice with 10 ml chloroform. PHB was precipitated by adding 180 ml methanol to the pooled chloroform phase. The pellet was dried to the air, dissolved in $CDCl_3$ to be analysed with H^1 and C^{13} NMR, the pattern compared to purified PHB.

NMR. H1 and C13-NMR-spectra were recorded using a JEOL 270GSX spectrometer (JEOL, Tokyo, Japan), equipped with a JEOL Stacman autosampler for 16 samples. Culture supernatant was analysed by adding 0.1 ml of D2O containing 3-(trimethylsilyl)[D4]propionic acid sodium salt (TMSP, 0.167 mM) to a 0.9 ml sample. The water signal was suppressed by irradiating this signal with standard NMR-software. The spectra were referenced using the TMSP signal at 0 ppm. Metabolites were quantified by integration of the relevant signals.

3. Results and discussion

Prior to experimenting with medium composition we tested whether the conditions in shake flasks were suitable for *B. pertussis* cultivations with respect to pH and dissolved oxygen, using MSS medium. The pH generally did not exceed 8.5, probably due to the low substrate concentrations used and the high buffer capacity of TRIS at alkaline pH. In all cases growth stopped after all glutamate and/or α-ketoglutarate had disappeared. In bioreactor experiments with the pH controlled at 7.2, similar yields in terms of growth rate and g C-$mole^{-1}$ were measured (data not shown). We therefore assumed that growth and yields were not significantly affected by the rising pH.

The dissolved oxygen tension was monitored qualitatively by adding 2 mg l^{-1} redox indicator resazurin to the cultures. The suspension remained colourless throughout the cultivation, indicating an aerobic environment. When the shaker was turned off while cells were in the late exponential phase, the cultures started to turn pink after 25 to 35 minutes. Since sampling 10 - 14 shake flasks generally required less than 15 minutes, we assumed that it had little effect on the aerobic metabolism of *B. pertussis.*

3.1 *B. pertussis* metabolism of various C-sources

A schematic summary of the catabolic and anabolic capabilities of *B. pertussis* is given in figure 1, combining the data generated by Jebb and Tomlinson (1951, 1955) and the data generated in this study. Figure 1 will be referred to throughout this paper, which is why it is introduced here. When a reaction can be considered anabolic (a) as well as catabolic (c), it is listed as catabolic in figure 1 (reactions 6, 7, 9, 10, 14). For simplicity, only reactions discussed in the text are numbered. Since *B. pertussis* can grow on glutamate and cysteine only, all major cellular building blocks such as amino acids, carbohydrates, fatty acids, and nucleotides can be synthesised from these two substrates. All anabolic routes in figure 1 can be deduced from this fact. Cysteine is excluded from figure 1 since it is present at a very low concentration as cystine, a less toxic dimer of cysteine (Jebb and Tomlinson, 1957). Therefore it probably serves as sulphur source only, not as energy source. A compound can be used in catabolism if its consumption is accompanied by the use of oxygen. For example, Jebb and Tomlinson (1951) found that glycine uptake was not accompanied by oxygen uptake (reaction 1a), which means that glycine is primarily used in anabolism. Alanine consumption (reaction 2c) on the other hand, is accompanied by an increase in oxygen uptake, which means that alanine can be used in anabolism as well as catabolism.

In order to identify carbon sources *B. pertussis* was grown with ammonium as an N-source and various C-sources (see legend figure 2 for details). The growth curves of the various cultures are shown in figure 2. Except for the culture with pyruvate, all cultures initially showed an increase in optical density, probably due to a carry-over effect from the preculture medium. Also, the breakdown of glutathion, a tri-peptide present in the supplement, consisting of glutamate, cysteine and glycine, may have been responsible for part of the initial growth. Only the media containing α-ketoglutarate or glutamate were able to sustain growth, all other media resulted in cultures that became stationary after approximately 18 hours.

Evidently, *B. pertussis* was able to convert α-ketoglutarate to glutamate (reaction 21c), but could not form glutamate from other glycolysis or citric-acid-cycle intermediates. In order to synthesize for example fatty acids from glutamate, *B. pertussis* must be able to convert oxaloacetate via pyruvate to acetylCoA (reactions 7 and 8 respectively). However, *B. pertussis* apparently cannot form α-ketoglutarate by condensing acetylCoA with oxaloacetate to form citrate, isocitrate and α-ketoglutarate consecutively (reactions 13, 15 and 18 respectively). Therefore, the citric-acid cycle can be considered as not functional in the sense that acetylCoA and oxaloacetate cannot be converted to α-ketoglutarate, thus growth ceases if no glutamate or α-ketoglutarate is present.

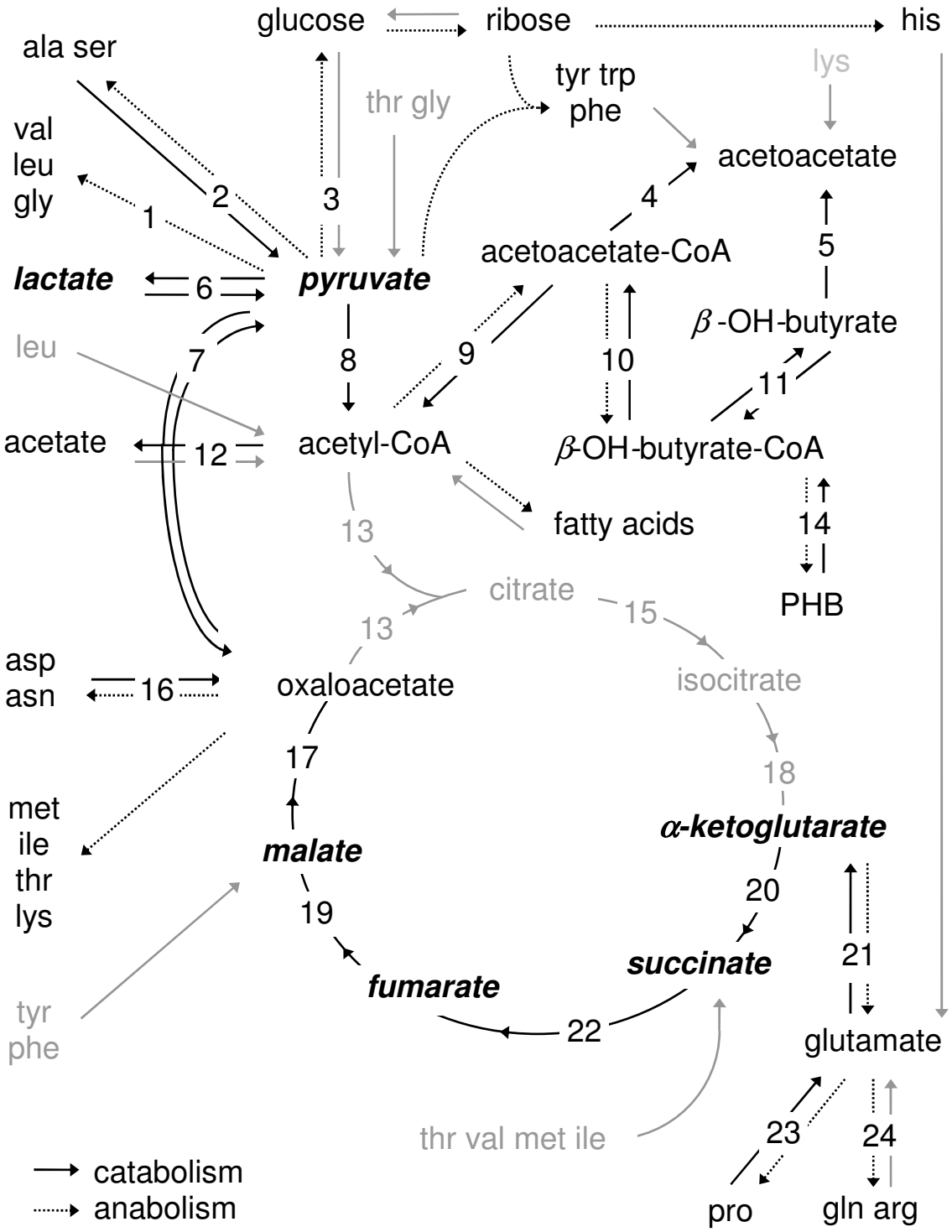

Figure 1. Overview of general metabolic pathways. Grey and black print: general metabolism (Michal, 1993), black print: deduced metabolic capabilities of *B. pertussis* (Jebb & Tomlinson, 1951, 1955) and this study. Bold italic print: substrates metabolised by *B. pertussis* that do not contain nitrogen.

The finding that *B. pertussis* does not have a functional citric-acid cycle is in conflict with Jebb and Tomlinson (1951) who reported that alanine, serine, proline and aspartate can be oxidised to carbon dioxide and water. Apparently, it is not possible to compare their measurements of oxygen uptake for a limited time with growth of bacteria over several days as studied here. In the follow-up experiments glutamate was used as N- and C-source, while various other C-sources were added to the medium.

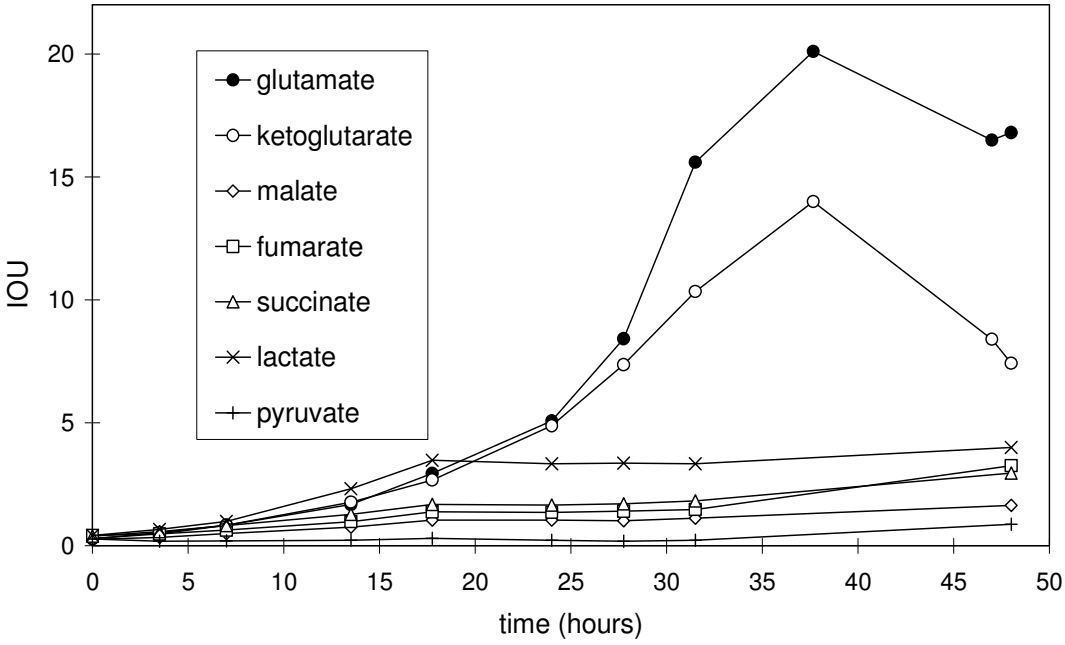

Figure 2. Cultures on media with 10 mM pyruvate, lactate, α-ketoglutarate, succinate, or fumarate added as the sole C-source, in the presence of 5 mM ammonium as N-source. Medium with 10 mM glutamate was included as a control. Each growth curve represents a single shake flask.

Next to the carbon sources used in figure 2, additional components were added to medium with glutamate. Cultures with added acetate, ethanol, glycerol, citrate, α- or β-glycerolphosphate all yielded growth curves and dry weights similar to the culture with glutamate only (data not shown), indicating that none of these compounds was metabolised to an appreciable extent. On the other hand, pyruvate, lactate, fumarate, succinate, and α-ketoglutarate were metabolised by *B. pertussis* in the presence of glutamate. The growth curves in media containing an N:C ratio of 1:8 with these carbon sources are shown in figure 3.

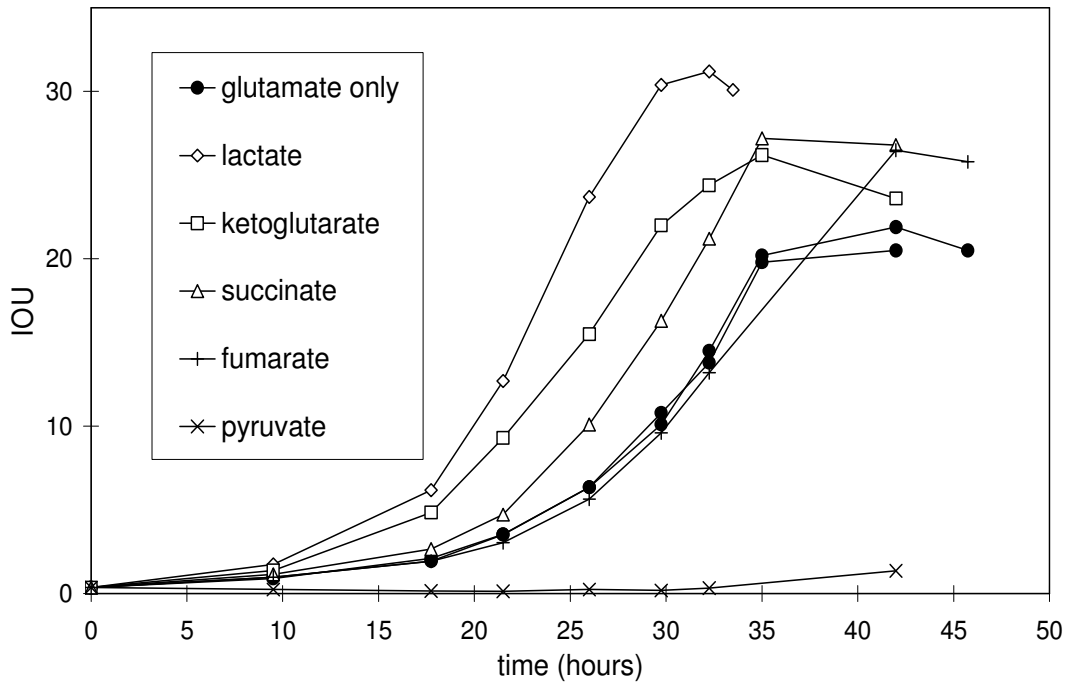

Figure 3. Cultures on media with a total of 100 mM C-atom, with 12.5 mM glutamate in all media. Two cultures with 12.5 mM glutamate without extra C-source added were used as controls. Each growth curve represents a single shake flask.

Except for the medium with glutamate and pyruvate, all carbon sources in combination with glutamate resulted in higher cell densities and less ammonium released per gram biomass formed than medium with glutamate only (data not shown). Cultures with the same carbon sources, yet at an N:C ratio of 1:12 also showed higher cell densities than cultures with glutamate only (data not shown). Apparently, the added C-sources were at least partly used in anabolism, presumably after conversion to either oxaloacetate (precursor of 6 amino acids) or pyruvate (precursor of 9 amino acids, all carbohydrates and fatty acids). Curiously, the growth rate in medium with pyruvate was much lower than in medium with glutamate only. In pyruvate-containing media with an N:C ratio of 1:12 and 1:20, i.e. concentrations of 19.4 and 25 mM pyruvate, *B. pertussis* repeatedly did not grow at all (data not shown). In an experiment to determine the order in which compounds were metabolised by *B. pertussis*, 5 mM of all intermediates used above were added to a basal medium containing ammonium. Pyruvate was consumed first, while lactate was produced (reaction 6), followed by consumption of α-ketoglutarate and lactate (data not shown). These findings indicate that although pyruvate can be metabolised very rapidly, a high pyruvate concentration does not result in a high growth rate. Assuming that pyruvate can diffuse into cells easily, it is possible that a high intra-cellular pyruvate concentration interferes with normal cellular metabolism or metabolic control mechanisms. Though interesting, we considered the matter outside the scope of this study, and concluded that pyruvate was not a suitable C-source.

The yields on media with glutamate and lactate or glutamate only was approximately 30% higher than the yields on media with α-ketoglutarate, succinate or fumarate in combination with glutamate (data not shown). The culture supernatants of medium initially containing α-ketoglutarate, succinate or fumarate in combination with glutamate contained β-hydroxy-butyrate (reaction 11c) as identified by two-dimensional NMR (data not shown). The formation of β-hydroxy-butyrate thus explains the lower biomass yield in these cultures compared to the yield on glutamate only or glutamate in combination with lactate. Therefore, we concluded that α-ketoglutarate, succinate, malate and fumarate were not suitable C-sources to balance the N:C ratio in *B. pertussis* media. After growth on Verwey, MSS and B2 medium β-hydroxy-butyrate was also found in the spent supernatant (data not shown). Since the formation of β-hydroxy-butyrate apparently occurs in all available *B. pertussis* media, the conditions governing its formation were investigated.

3.2 Excretion of metabolites and waste metabolism

Usually incomplete oxidation of substrates occurs when these are present in high concentrations. The Crabtree effect, i.e. the anaerobic degradation of glucose by yeast under aerobic conditions, is a well known case of incomplete oxidation caused by high substrate concentrations. Here we focussed on the concentration of glutamate and the addition of α-ketoglutarate as model compound known to cause β-hydroxy-butyrate excretion. The influence of substrate concentration on biomass yield was investigated by reducing the substrate concentrations stepwise to 1/2, 1/4 and 1/8 of the undiluted concentrations of α-ketoglutarate and glutamate. The dry weight determined in duplicate multiplied with the dilution of the substrates should be the similar for all dilutions if substrates are completely metabolised. This was not the case since the dry weights corrected for the dilution factor averaged 1.7±0.3 g l^{-1} in all diluted media (n=2x3) combined, compared to 1.1±0.1 g l^{-1} in the undiluted medium (n=2).

NMR performed on the culture supernatants revealed how *B. pertussis* metabolism changed during the cultures. An example of an NMR spectrum is given in figure 4, of the undiluted medium after 23 hours of growth. In the undiluted medium a range of compounds containing carbon-hydrogen bonds were detected: TRIS, glutamate, and α-ketoglutarate. Next to these medium components a considerable amount of succinate was formed during the cultivation. Minor amounts of formate, fumarate, malate, acetate and ethanol (visible in figure 4) and traces of lactate and valine were also detected (not visible in figure 4).

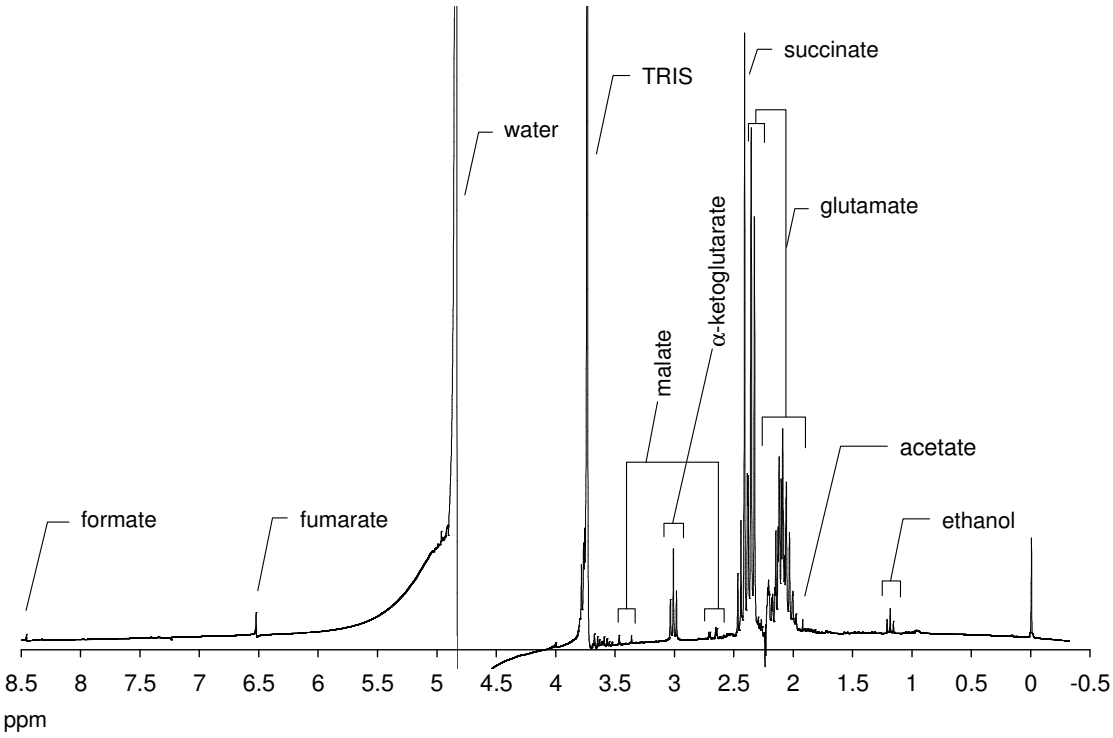

Figure 4. NMR spectrum of spent undiluted medium that initially contained 14.3 mM α-ketoglutarate and 28.5 mM glutamate after a cultivation of 23 hours.

The consumption and production of various metabolites during the cultures in the undiluted and two-fold diluted media as measured with NMR, are shown in figures 5A and B, respectively. In both media a large amount of succinate was formed during the first 16 to 24 hours, which was consumed again later in the culture. The same pattern was observed for fumarate and malate (not shown in figure 5). Apparently these substrates are readily excreted and taken up by the bacterium. Therefore, the observed dysfunctional citric-acid cycle is not the result of a reduced permeability of the bacterium for these substrates. After most of the succinate was consumed, β-hydroxy-butyrate and acetate were produced in undiluted medium (reactions 11c and 12c respectively). In two-fold diluted medium, no acetate was formed, whereas the final concentration of β-hydroxy-butyrate was approximately one fifth of the concentration in the undiluted medium. At the end of the culture with undiluted medium, all succinate, fumarate and malate had disappeared, while β-hydroxy-butyrate and acetate had not.

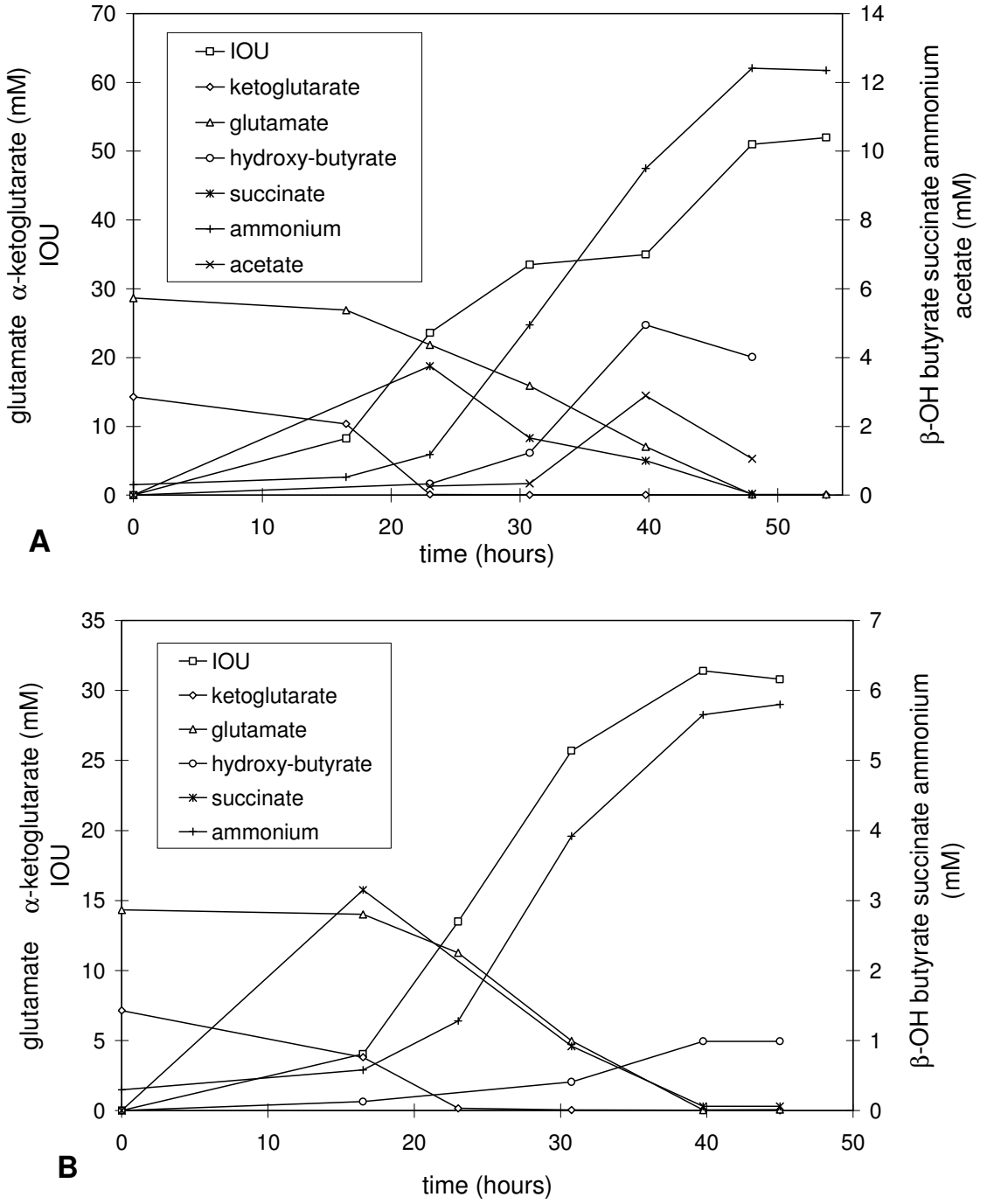

Figure 5. Consumption and production of various metabolites during cultivation of *B. pertussis* in A: undiluted and B: two-fold diluted medium (Y-axes of figure B are half of those of figure A).

The yields on 10 mM α-ketoglutarate only was 7.3 g C-mole^{-1} whereas the yield on 5, 8.3, 10 and 12.5 mM glutamate averaged 8.7±0.10 g C-mole^{-1}, in all cases without detectable metabolites during or at the end of the culture. The yield on 60 mM glutamate, the concentration in MSS medium, was 6.2±0.4 g C-mole^{-1}, while acetoacetate and β-hydroxy-butyrate were present at the end of the culture. In other words, the combination of 7 mM α-ketoglutarate and 14 mM glutamate (fig.

5B) caused *B. pertussis* to form some β-hydroxy-butyrate, whereas the individual components at low concentrations (~10 mM) did not. Higher concentrations of both substrates also resulted in excretion of acetate. Acetate, or citric-acid-cycle intermediates were not detected during the cultures on 60 mM glutamate alone, indicating that α-ketoglutarate was responsible for the excretion of acetate and citric-acid-cycle intermediates.

The biomass determinations above were not 'true' biomass yields in the sense that maintenance was corrected for, yet as an approximation the values can be compared with available literature data. Licari *et al.* (1991) found a 'true' biomass yield of 5.8 g C-mole^{-1} on glutamate at an initial concentration of 91 mM. Using the same medium, Frohlich *et al.* (1995) found a yield of 6.73 to 7.44 g C-mole^{-1}. Next to glutamate, 3 g l^{-1} acid-hydrolysed casein was also present in their medium, also responsible for part of the biomass yield, so that the actual biomass yield on glutamate of Frohlich *et al.* (1995) is probably lower than 6.73 g. C-mole^{-1}. The yield reported by Andorn *et al.* (1988) on MSS medium corresponds to 6.7 or 7.0 g/C-mole^{-1} depending on whether or not proline is taken into account. These values agree better with 6.2 g C-mole^{-1} found for 60 mM glutamate than with 8.7 g C-mole^{-1} for low concentrations of glutamate.

Though the formation of β-hydroxy-butyrate explained the lower biomass yields, it was unclear why it was formed towards the end of the culture, instead of during the first part of the culture when substrate levels are high. Furthermore, the N:C consumption ratio in figure 5B after 15 hours of cultivation was 1:30, whereas it was 1:12 after 30 hours of cultivation (data not shown). Since the composition of cellular components is usually fairly constant, we suspected the formation of a nitrogen-free storage material in cultures that excreted β-hydroxy-butyrate at the end of the culture. Therefore cells grown on B2, and MSS medium were analysed during various growth stages with Electron Microscopy (EM). Since no β-hydroxy-butyrate was formed on medium with 10 mM glutamate and 16.7 mM lactate, it was included as a 'negative control'.

EM showed that cells in the early exponential phase grown on B2 medium contained 3 to 5 globules per cell (figure 6A). Cells grown on MSS medium contained 1 or 2 globules per cell (figure 6B), while only occasionally a globule was seen in cells grown on glutamate and lactate medium (figure 6C). In the late logarithmic phase the globules disappeared, coinciding with the appearance of β-hydroxy-butyrate, alone or in combination with acetoacetate in the case of MSS medium (data not shown). Since β-hydroxy-butyrate and/or acetoacetate appeared as the globules disappeared, it seemed logical that the globules consisted of polymerised β-hydroxy-butyrate, i.e. poly-hydroxy-butyrate (PHB), a common biopolymer (reaction 14a). Using an isolation procedure for PHB we

were able to confirm using C^{13} and H^{1} NMR that the globules indeed consisted of PHB (data not shown).

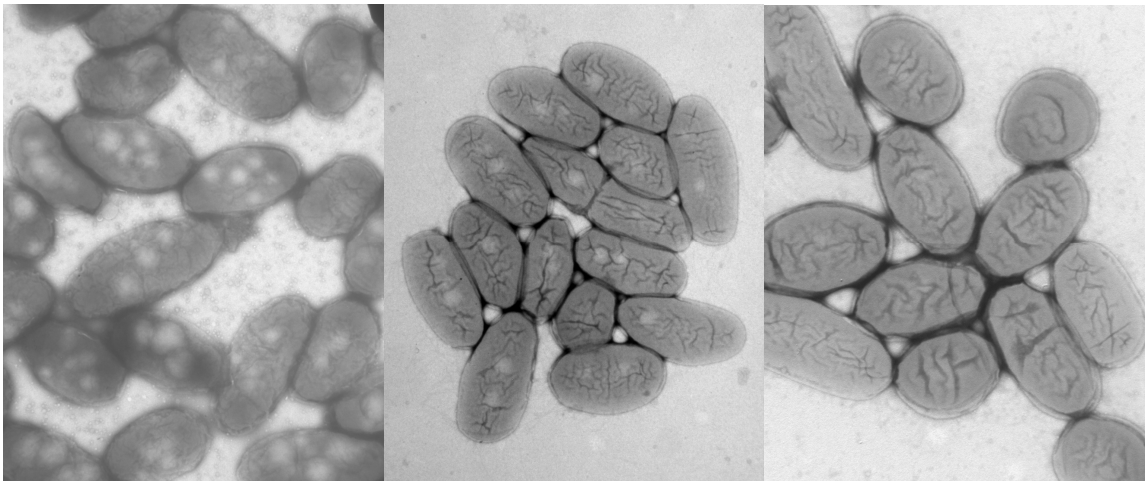

Figure 6. *B. pertussis* cells taken during the early exponential growth phase, stained with PTA, from left to right cultured on; B2 medium, Stainer Scholte medium, medium 10 mM glutamate and 16.7 mM lactate. The cells of these cultures were taken at comparable densities.

Although very few globules were seen in cells grown on glutamate and lactate, a substantial amount of PHB was also isolated from these cells during the early logarithmic growth phase, yet no β-hydroxy-butyrate was excreted during the culture. Although PHB was formed, it could apparently be rerouted back into the main metabolism. In an experiment where β-hydroxy-butyrate was added to medium with glutamate and lactate, no net consumption of β-hydroxy-butyrate was observed, although 20% was converted to acetoacetate after all lactate and glutamate were consumed (data not shown). Rerouting free β-hydroxy-butyrate into main metabolism requires condensation with CoA (reaction 11a), at the expense of 1 ATP. Apparently this is energetically unfavourable in the presence of lactate and glutamate that can directly be used to generate energy. The finding that free β-hydroxy-butyrate was not metabolised while PHB can be metabolised without β-hydroxy-butyrate excretion suggests that β-hydroxy-butyrate is probably released from PHB in the form of β-hydroxy-butyrate-CoA (reaction 14c). The latter compound can be dehydrogenated by NAD and thereafter thiolysed by CoA, yielding 2 acetylCoA molecules (reaction 10c and 9c respectively). Since *B. pertussis* cannot oxidise acetylCoA, the rate at which acetylCoA units can be used in anabolism is limited. Probably any excess β-hydroxy-butyrate-CoA is hydrolysed, while generating 1 ATP, also regenerating CoA in the process.

The data above lead to the following hypothesis regarding the role of PHB in metabolism of *B. pertussis*. During exponential growth, at high substrate concentrations, more acetylCoA is generated than can be utilized in anabolism. In order to maintain its metabolism, CoA needs to be regenerated. This can be

achieved by hydrolysis of acetylCoA to acetate and CoA (reaction 12c), generating 1 ATP. Alternatively, by condensing 2 molecules of acetyl-CoA, acetoacetate-CoA can be formed, releasing 1 CoA (reaction 9a). Acetoacetate-CoA can in turn be converted to β-hydroxy-butyrate-CoA using one NAD(P)H in the conversion (reaction 10a). The latter reaction is only useful if it serves to regenerate NAD(P) and/or when β-hydroxy-butyrate is polymerised to PHB (reaction 14a), releasing the second CoA molecule. Once external substrates have become less available PHB will be metabolised together with the remaining substrates. The total PHB quantity present per bacterium determines whether all PHB can be converted into biomass or whether the monomer will be excreted. Probably, the rate at which β-hydroxy-butyrate is formed determines whether it is converted to acetoacetate or not, since *B. pertussis* can only partially oxidise β-hydroxy-butyrate to acetoacetate. The formation of PHB allows *B. pertussis* to continue growing while glutamate or α-ketoglutarate concentrations are diminishing. Once glutamate or α-ketoglutarate has disappeared, the organism may also be able to maintain its viability by hydrolysing PHB. The control mechanisms of the PHB metabolism are outside the scope of this study and were not investigated further. For practical purposes we concluded that intra-cellular PHB formation is only a problem if it results in lower biomass yields due to β-hydroxy-butyrate or acetoacetate excretion.

Since Jebb and Tomlinson (1951) did not include glutamine in their studies, we attempted to cultivate *B. pertussis* on glutamine as sole amino acid in the basal medium in order to complete figure 1. Repeatedly no growth occurred, which was somewhat unexpected since glutamine can be degraded to glutamate in one reaction step. Goldner *et al*. (1966) and Vajdic *et al*. (1966) reported growth on a mixture of glutamic acid and glutamine indicating that glutamine can be taken up and incorporated into biomass. The fact that no growth occurred on media with glutamine only suggests that although glutamine can be used in anabolism, it cannot be catabolized by *B. pertussis.*

3.3 Nitrogen:Carbon balancing of *B. pertussis* medium with lactate

Since lactate did not cause metabolite excretion, its use as a means to reduce the accumulation of ammonium was investigated more quantitatively. To determine the consumption N:C ratio lactate and glutamate were mixed in various ratio's while ammonium was added to prevent nitrogen limitation resulting in media with an N:C ratio of 1:10, since this was the N:C consumption ratio found for cultures on glutamate only (data not shown). The data summarized in table I shows that none of the cultures was nitrogen limited, since there was always ammonium present at the end of the culture. No metabolites were excreted during the cultivations, even though in some of the media significant amounts of

lactate were present after growth had stopped. More significantly, table I shows that when sufficient lactate is present, net consumption of ammonium takes place instead of excretion. This observation is significant, since it confirms that *B. pertussis* can indeed redistribute all surplus ammonium from glutamate to other cellular compounds synthesised from lactate, thus avoiding ammonium accumulation altogether.

Table I. Metabolite concentrations and calculated parameters the cultures grown on various ratios of glutamate to lactate to ammonium, resulting in media with an N:C ratio of 1:10. Results are the mean of duplicate cultures, metabolites measured in duplicate, reported with the standard deviation of the mean.

composition [a]) (mM)					**calculated parameters**	
medium			**spent supernatant**			
glutamate	**NH_4^+**	**lactate**	**NH_4^+**	**lactate**	**NH_4^+ excreted[b]) (mmol.g^{-1} biomass)**	**Yield g. C-mole^{-1}**
0.5	9.5	32.5	8.0	24.5	-6.9	7.7
1	9.0	31.7	7.5	22.1	-5.2	8.2
2	8.0	30.0	6.3	17.4	-4.0	8.5
5	5.0	25.0	3.2	8.8	-2.5	9.4
10	-	16.7	1.5	0.7	1.9	7.9
10	-	-	4.6	0.00	10.9	8.2

[a]) Glutamate present as glutathion (0.32 mM) and unconsumed lactate were taken into account when determining the yield and the glutamate to lactate consumption ratio. Glycine, cysteine, ascorbic acid, nicotinic acid were not corrected for.
[b]) Expressed as: ($[NH_4^+]_{final} - [NH_4^+]_{initial}$).(gram dry weight)$^{-1}$. Negative values correspond to consumption of added NH_4^+.

The transfer of ammonium to a carbon skeleton usually requires the input of 1 NAD(P)H and 1 ATP. The conversion of lactate to a central metabolite such as pyruvate only yields one NADH, while the conversion of glutamate to pyruvate yields 3 NADH, 1 FADH and 2 ATP. Assuming a conversion efficiency of 1 NADH to 1 ATP the energy yield of *B. pertussis* on glutamate is roughly 6-fold higher than on lactate. Therefore, we expected a considerable decline in yield as the ratio glutamate:lactate increased. Table I however shows little indication that cultures growing primarily on lactate do not generate enough energy to carry out all anabolic functions. An intriguing question is what happens to the surplus energy generated when the organism grows entirely on glutamate.

At physiological pH glutamate has a net negative charge of –1. When consumed, its charge is transferred to water, creating OH^{-1}, resulting in a pH-rise. *B. pertussis* can utilize glutamate:lactate ratios of more than 1:1. Lactate can be added during the culture in the acidic form, compensating the rise in pH.

Consequently, in a fed-batch system, lactate can be used as a means to completely compensate the rise in pH caused by the consumption of glutamate instead of an acid like HCl, which increases the osmolarity and eventually hampers growth (Frohlich *et al.*, 1995).

4. Concluding remarks

On the basis of theoretical considerations and simple shake flask experiments a balanced medium was designed consisting of lactate and glutamate as main substrates, in which ammonium does not accumulate. The partly dysfunctional citric-acid cycle, *B. pertussis'* waste metabolism and its ability to synthesize and (de)polymerise β-hydroxy-butyrate were discovered as results of these straightforward experimental procedures. Although some PHB is formed during the cultivation of *B. pertussis* on lactate/glutamate medium, no β-hydroxy-butyrate is excreted, ensuring an efficient conversion of carbon to biomass, unlike current cultivation media for *B. pertussis*.

At this point only very straightforward and simple experiments were done; no virulence-factor production was measured. Preliminary experiments indicate that the production of FHA and PT per gram biomass on medium with 16 mM lactate and 10 mM glutamate is higher than on MSS medium. More quantitative data will be reported later, as well as the influence of the medium matrix on virulence-factor production.

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CHAPTER 3

Effect of relevant culture parameters on Pertussis Toxin expression by *Bordetella pertussis*.

Summary

Whooping cough vaccines are produced using different ranges of cultivation conditions and medium compositions, which are known to influence growth rate, virulence factor production and degradation, as well as the virulence factors' association to the cell. This study quantifies the impact of individual parameters on Pertussis Toxin (PT) production, using an optimised chemically-defined medium as starting point, rather than a complex medium. A number of chemicals are identified that affect both growth rate and virulence factor production, which occur at similar levels in various commonly used production media. Also, degradation by proteolytic activity is shown to be an important parameter to monitor, since it significantly affects the PT yield. Low sodium concentrations, i.e. 50-75 mM rather than the conventional 100-140 mM, significantly increase the growth rate of the organism, the final optical density, as well as the association of PT to the cells. The absolute amount of biomass produced measured as dry weight, is similar for all sodium concentrations tested, contrary to earlier work. While it is known that high iron concentrations inhibit virulence factor production, it is shown here that iron-limited growth results in very high specific PT production. This finding may be used to produce a whole-cell vaccine with little biomass per dose, reducing whole-cell vaccine toxicity. The *B. pertussis* strain 509 used here produces 30% more PT at 34 than at 37°C, a commonly used cultivation temperature. The data in this study show that existing production processes for cellular and acellular vaccines can in principle be optimised considerably by taking simple measures.

Key words: Pertussis Toxin production, vaccine, medium

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1. Introduction

Whooping cough is caused by the Gram-negative bacterium *Bordetella pertussis* and *B. parapertussis,* a highly contagious disease that mainly affects infants and young children. Most developing countries use inactivated whole cells of *B. pertussis* as vaccine. The production process of such a cellular vaccine is straightforward and low-cost; cells are cultivated, concentrated and inactivated by exposure to heat and/or chemicals. However, the lipo-poly-saccharide (LPS) present in *B. pertussis'* outer membrane causes adverse reactions after vaccination such as fever. Therefore, the western world has largely switched to the more expensive acellular vaccines, which cause less adverse reactions. These vaccines consist of 1-5 *B. pertussis* protein virulence factors. In order to produce an acellular vaccine, the cultivation step is followed by extensive purification procedures.

For both vaccine types a high yield of virulence factors per unit biomass produced is desirable, since more vaccine doses per production run can be obtained leading to lower costs. For cellular vaccines a higher yield of virulence factors per unit biomass is even more important, since the amount of LPS per dose can be lowered, which is likely to result in less adverse reactions. In addition to a high yield, it is important for cellular vaccines that all virulence factors produced remain cell associated, since the culture supernatant is discarded. Although especially Pertussis Toxin (PT) is prone to dissociate into the supernatant during the cultivation, the parameters that influence the association of PT to the cells during cultivation have not been studied.

The PT content in an acellular vaccine is 5 to 25 fold higher than in a cellular vaccine when the composition of various acellular vaccines [1] and the amount of PT in a cellular vaccine [2] are compared. The other virulence factors, such as filamentous hemagglutinin (FHA), pertactin (PRN) and fimbriae (FIM), occur in 2 or 3-fold higher concentrations in acellular vaccines than in cellular vaccines. Hence, much more PT needs to be produced than any of the other virulence factors, which makes PT production the target to optimise in acellular vaccine production.

The expression of PT and other virulence factors is controlled by the bordetella virulence gene (*bvg*) locus (Fig. 1A). Virulence starts with phosphorylation of the intracellular protein BvgA by the membrane protein BvgS in response to environmental signals (Fig. 1B). Phosphorylated BvgA stimulates expression of FHA, Fim, BvgS and itself. Next, as the amount of BvgS and phosphorylated BvgA rises, the expression of all virulence factors is stimulated, including PT (Figure 1C). Although high BvgA levels stimulate expression of all virulence factors, additional factors may be involved, co-regulating and attenuating the regulation by the bvg locus as suggested by several authors [3,4].

A number of chemicals called modulators have been identified that negatively influence virulence factor expression [5, 6, 7]. Most modulators, like nicotinamide, Ca^{2+}, NH_4^+, and Fe^{3+}, do not occur in the host organism at the concentration required to modulate virulence factor expression, nor are the mechanisms understood how these factors influence the *bvg*-locus. However, they may be of importance for vaccine production in bioreactors as most of these compounds occur in commonly used cultivation media. Most of the work involving modulators was, however, aimed at elucidating rather

than quantifying the mechanisms involved. Therefore, usually only the extremes of modulation were investigated, generally using culture conditions like agar plates [6,7], which are inhomogeneous and therefore not representative for production conditions.

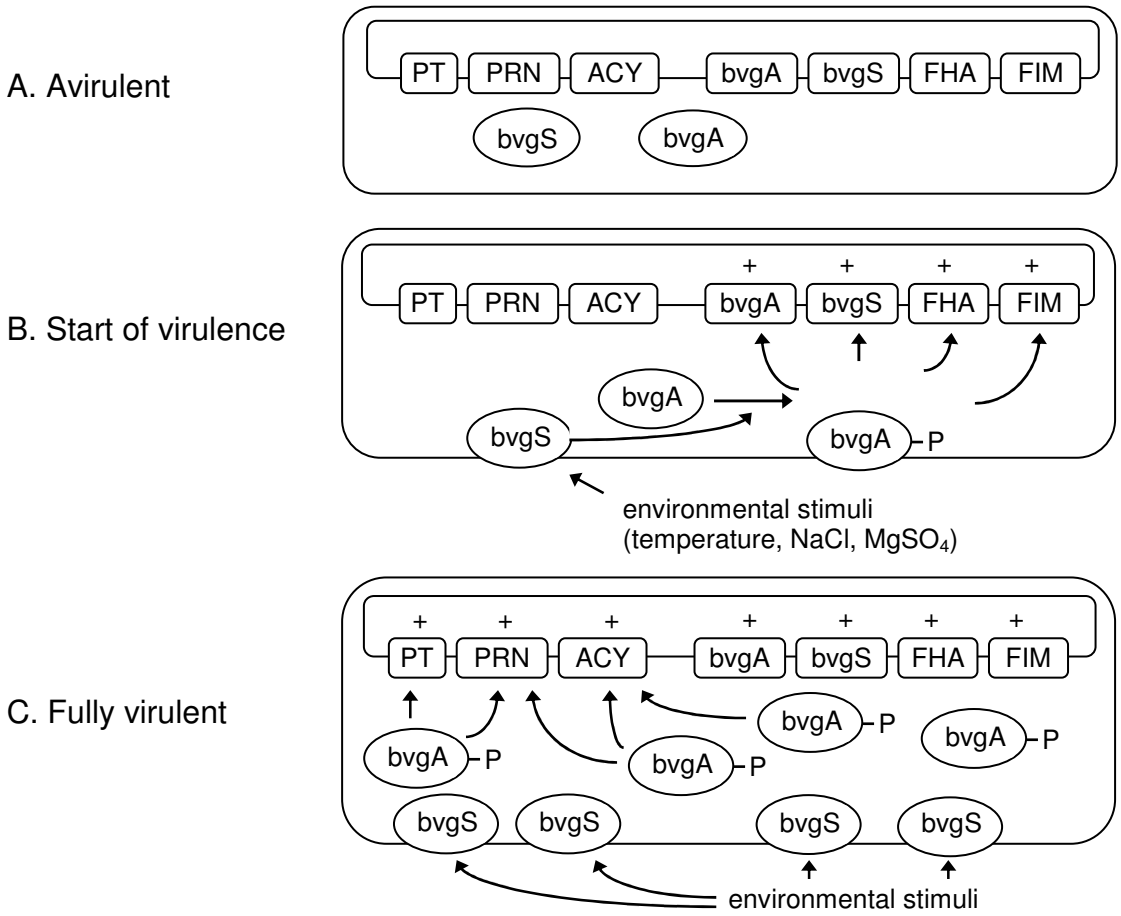


Figure 1. Regulation of *B. pertussis* virulence-factor expression. Abbreviations; PT-pertussis toxin, PRN-pertactin, ACY-adenylate cyclase, BvgA -*bvg* regulated activator protein, BvgS - *bvg* regulated sensor/BvgA phosphorylating protein, FHA-filamentous heme-agglutinin, FIM-fimbriae. A: avirulent, corresponding to an environment outside the host-organism, B: start of virulence, the situation during the first hours after infection of a host, C: fully virulent, all virulence factors are expressed, several hours after infection of a host.

Next to production of virulence factors and their association to the cell, it is also necessary to look at degradation of virulence factors during cultivation. Taxonomically, *B. pertussis* is classified as protease negative, since gelatin plates show no liquification around colonies. However, degradation of FHA has been reported [8] as well as the proteolytic degradation of subunit 1 and 5 of PT [9]. The Phe-Asp sequence that is recognized by the *B. pertussis* protease described by Cyr *et al.* [10] occurs 7 times in FHA, once in PT, once in PRN and twice in the fimC protein. The effect of *B. pertussis'* protease during cultivations has not been studied before, but may have quite an impact on the virulence-factor content and may complicate the interpretation of experimental results.

In conclusion, for a number of culture parameters the effect on virulence factor expression and their association to the cell is not properly quantified for production processes. In addition, the effect of proteolytic activity during the cultivation is unknown. Therefore, the aim of this paper is to properly quantify the effect of a number of parameters on virulence factor expression, their association to the cell, and the effect of these parameters on proteolytic activity. From Figure 1C it is clear that if PT is formed, all other virulence factors are also expressed. Therefore, PT production is used as a measure for virulence factor production.

As a starting medium, the chemically-defined medium developed by Thalen *et al*. [11] was used rather than any of the complex production media or the chemically-defined Stainer Scholte medium [12]. The latter media lead to the formation of large amounts of ammonium and poly-hydroxybutyrate, which can influence virulence factor production. In the medium developed by Thalen *et al.* [11], only minor amounts of these compounds are formed. Furthermore, growth on this medium is far more reproducible than growth on a complex production medium. On the basis of the data generated, it should become clear what the impact of each single parameter is on *B. pertussis*' virulence factor production. The implications of the effects found for the different parameters (Table 1) for the improvement of existing production processes will be discussed.

2. Materials and methods

Media. The Modified Stainer Scholte medium [12], modified further by Thalen *et al.* [11], resulting in the THIJS-medium, with an N:C ratio of 1:10, a Na^+ concentration of 75 mM and a Fe^{3+} concentration of 40 µM was used as a standard in all experiments and served as reference culture for the experimental cultures.
Strain, growth conditions. Strain 509 was used in all experiments; it is one of the two strains included in the DPT-IPV formerly produced by the NVI in the

Netherlands. Growth conditions and experimental equipment were described before [11]. Briefly, pre-warmed 0.5 L bottles containing 200 ml medium were inoculated with a 5% inoculum from a -70 ^{o}C stock and incubated for 24 hours to an OD of 1.6 - 2.0. This culture was used to inoculate the experimental cultures using a 1% inoculum. Bottles were incubated at 35 ^{o}C, on a rotary shaker at 200 RPM. For all experimental conditions, cultures were done in duplicate. All samples were analyzed individually. Results are presented with the standard deviation of the mean of both cultures.

PT and proteolytic-activity determination. The enzymatic method of Cyr *et al.* [10] was used. Briefly, the method employs a synthetic fluorescein-labeled oligo-peptide that is ribosilated by PT. The ribosilated oligo-peptide is separated from the non-ribosilated peptide on HPLC, using peak area as a measure for PT activity. In order to compensate for matrix effects on PT activity, samples are measured with and without a known amount of PT added. The PT standard JNIH90/518 was used as a reference. Proteolytic activity was determined by quantifying the second peak on the chromatogram, i.e. the degraded oligo peptide. This peak is the result of the proteolytic activity during the incubation. Proteolytic activity was largely avoided by adding protease inhibitor PMSF to culture samples meant for PT determination, directly after these were taken and stored at -20 ^{o}C. Samples were either 0.22 µm filtered (supernatant), or directly stored at -20 ^{o}C (suspension). No PMSF was added to supernatant and suspension samples taken for proteolytic activity determination. Every sample taken was assayed in duplicate, both for PT as well as for proteolytic activity.

Biomass determinations and yields. The optical density of the suspensions was measured on a Vitalab 10 (Vital Scientific, Dieren, the Netherlands), at 590 nm. Dry-weight determinations using the entire content of a shake flask, were done as described before [11]. The culture dry weight was determined using the entire remaining culture volume of each culture, after the optical density did not increase further. The PT yield was calculated by dividing the highest amount PT measured during an experimental run by the dry weight at the end of that culture. Usually this time point corresponded with the highest OD590. The PT yields of the cultures were related to the PT yields of the THIJS medium that was always included in every experiment. The THIJS medium was tested in quadruplicate in a bioreactor at a dissolved oxygen level of 20% saturation with constant agitation yielding 2.03 ±0.47 mg PT/g biomass. The PT yield on the THIJS was set to 2.0 mg/g biomass, recalculating the PT yield of the shake bottle cultures relative to this number.

3. Results and Discussion

In a first study the effect of the parameters listed in Table 1 on PT production was quantified using an antigen binding ELISA method [18]. On the basis of these experiments an optimal medium was formulated, the THIJS medium. However, in this first study it became clear that the ELISA was not accurate enough to determine moderate differences in PT production. In addition, the method could not discriminate between intact PT and PT degraded by proteases. Therefore, the complete study was repeated using the new method of Cyr *et al.* [10], which measures the enzymatic activity of PT and had just become available. THIJS medium was used as a starting point, at a temperature of 35 ^{o}C. For each parameter studied, the THIJS medium was included. Only the results of the last study are shown here.

Table 1. Parameters for which the effect on proteolytic activity, production of PT and its association to the cell is studied.

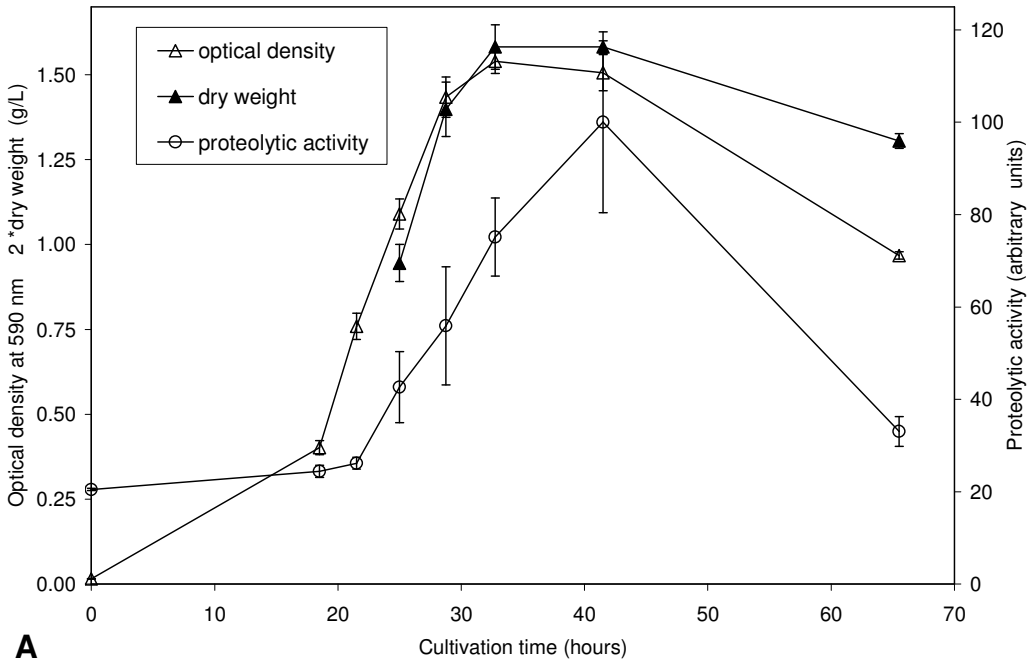
Parameter	**Rationale**
Glutathione	Contains a free -SH group that may be toxic [13], just like the free -SH group in cysteine. In addition, oxidation of cysteine to sulphate by *B. pertussis* can result in inhibition of virulence-factor expression [14].
Nicotinic acid	Is a known modulator of virulence factor expression at 4 mM, but the effect of lower concentrations has never been studied in suspension culture.
TRIS buffer	Is known to destabilize membranes of gram-negative organisms [15]. Likewise, TRIS' destabilization of membranes may lead to dissociation of virulence factors from the outer membrane of *B. pertussis*.
N:C ratio	The N:C ratio has quite an impact on growth and byproduct formation [11], which may also affect the virulence factor production.
Sodium	High sodium concentration lowers the growth rate, but stimulated PT production [16]. However, the association of PT to the cell and the effect of proteolytic activity was not studied before.
Iron	High [Fe^{3+}] causes low virulence-factor expression [5]. While total lack of iron will limit growth, low [Fe^{3+}] might increase PT expression.
Temperature	Temperatures of 27 ^{o}C or less result in inhibition of virulence-factor expression [17]. While *B. pertussis* is cultivated at a range of different temperatures, no clear optimum was ever established for this parameter.

3.1 PT production and proteolytic activity

In order to standardize experimental procedures, 10 cultures were grown under identical optimal conditions as mentioned above. During the culture samples were taken for OD, PT and proteolytic activity, while at various stages cultures were harvested two by two to determine dry weight. The biomass formation and protease and PT activities during the culture are shown in Figure 2. The optical density stopped increasing after 33 hours, and remained fairly constant for 9 hours. In the same period however, the PT concentration in the suspension and the supernatant declined to less than 60% and 40%, respectively, of the maximum concentration. Therefore, in all following experiments samples around the late exponential phase were taken every 2 to 4 hours, to avoid missing the maximum PT concentration as a consequence of degradation.

Judging from the rising proteolytic activity during the growth phase of the culture, and the decline of PT activity after production stopped, PT is both formed and degraded during the cultivation. However, 24 hours after the maximum, the proteolytic activity decreased to 35% of the maximum, indicating that the protease is not very stable at the cultivation temperature of 35 °C. Though degradation of PT and possibly other virulence factors definitely requires further study, it was considered outside the scope of this study. Here, the proteolytic activity was monitored in order to make a rational choice between maximal PT production and minimal proteolytic activity.

The finding that PT production ceased after growth has stopped agrees with the findings of others [16, 8] who reported that PT production stopped when *B. pertussis* enters the stationary phase. The finding that PT is degraded to an appreciable extent during the cultivation was not shown in previous studies, which also reported on cultures that continued well into the stationary phase [16,8]. Degradation of PT may not have been observed by these authors, since an ELISA was used to measure the PT production rather than the enzymatic activity of the protein. Loss of PT's enzymatic activity, which has been reported during purification, but not during cultivation, means loss or degradation of the enzymatically active subunit 1. The significance of this finding is that most of the antibodies *in vivo* are directed against the enzymatically active subunit 1. Consequently, loss of subunit 1 will alter the immunogenic properties of a cellular or acellular vaccine, possibly affecting vaccine efficacy.



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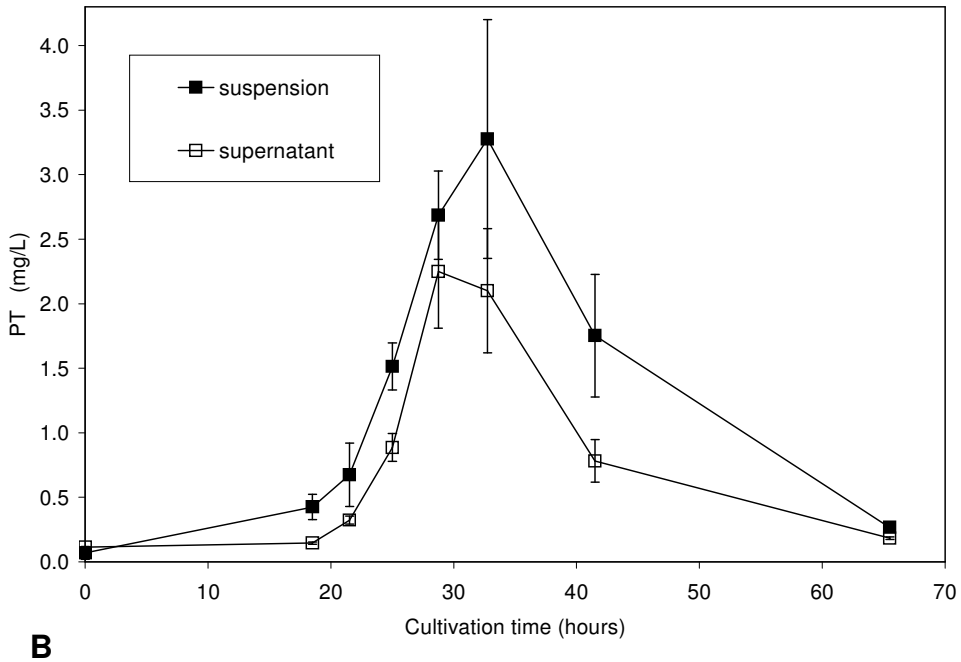

B

Figure 2. Optical density at 590 nm, dry weight and proteolytic activity (2A), and the PT concentration in both the total suspension and supernatant, (2B) of 10 cultures grown under identical circumstances. After 18 hours, samples for OD and PT were taken from all 10 cultures. After 25 hours this was repeated and the complete culture volume of 2 of the 10 cultures was used for a dry weight determination. The remaining 8 cultures were left to grow until the next time point, at which all bottles were sampled for OD and PT, and 2 more cultures were used for a dry weight determination, and so on until all bottles were used. Therefore the average PT and OD values are the average of all cultures present at that time point, while the dry weight value is the average of 2 cultures. The error bars represent the standard deviation of all values measured at that time point.

3.2 Effects of Tris, nicotinic acid, glutathione and N:C ratio on PT production

Replacing, omitting or changing the first 4 parameters in Table 1, i.e. Tris, nicotinic acid, glutathione and N:C ratio did not result in an improvement of total PT production or the association of PT to the cell and are therefore only briefly discussed here:

- Replacing TRIS by glycerol-phosphate [19] or omitting TRIS entirely, did not result in an increased association of PT to the cell (results not shown).
- Replacement of the modulator nicotinic acid by the non-modulating compound anthranilic acid [7], had no effect on PT production or its association to the cell (results not shown).
- Omitting the potentially toxic glutathione resulted in a clearly lower PT production (no data shown). This could be due to the lack of the amino acids in glutathione, although there are no data on metabolic breakdown of this compound. For glycine this was tested by supplementing the medium with this amino acid. However, adding 0.3 mM glycine instead of glutathione resulted in a 50% reduction of growth rate (no data shown). Glycine is present in all complex production media, either as part of acid hydrolyzed casein or as part of yeast extract (Quest, product catalog). Based on an average extract or hydrolysate addition of 5 to 10 gram/liter, the free glycine content of complex production media will be at least 1 to 3 mM, i.e. significantly higher than the concentration added here. Though not affecting PT production directly, apparently glycine can cause a significant reduction in growth rate, and thus affects bioreactor productivity.
-The medium N:C ratio of 1:10 was primarily chosen by Thalen *et al.* [11] to ensure that NH^{+}_{4} would not accumulate. In the range of an N:C ratio from 1:5 to 1:20, the PT production and its association to the cell was similar from an N:C ratio 1:8 to an N:C ratio of 1:12 (no data shown).

As a result of the experiments indicated above, the medium used in all experiments contained TRIS, had an N:C ratio of 1:10, and received a supplement containing glutathione and nicotinic acid, i.e. the composition of the THIJS medium.

3.3 Na^{+} concentration

The growth rate of *B. pertussis* decreases with increasing Na^{+} concentrations [16]. This finding is confirmed in this study as shown in Figure 3a. Not only did the [Na^{+}] profoundly affect the doubling times, but also the maximum optical density. The maximum optical density for cultures with 25 or 50 mM Na^{+} was approximately

80% higher than the optical density for cultures with 175 mM Na^+. However, the final average dry weights of all the cultures in Figure 3a had the same value of 0.82 ± 0.062 g/L (no data shown). Based on OD data only, it would appear as if the biomass yield on the same medium increased with decreasing sodium concentrations. The combination of measuring OD and dry weight used here, shows that merely the optical characteristics of the cultures were influenced by the sodium content, not the biomass yield.

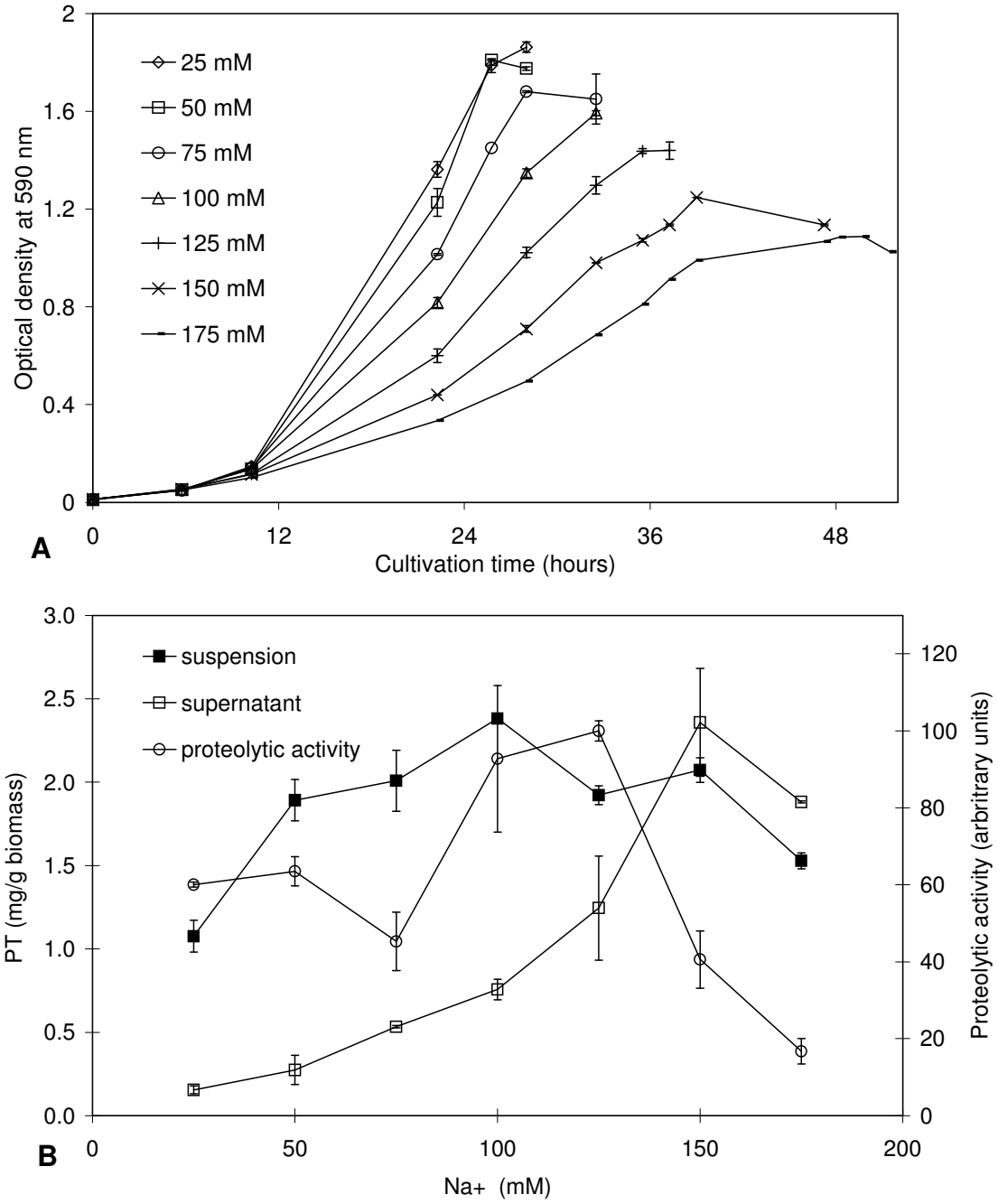

Figure 3. Growth curves (3A) and PT production (3B) of duplicate cultures in media containing 25 to 175 mM Na^+. The average dry weight of all cultures was 0.82 ± 0.062 g/L. The error bars represent the standard deviation of samples from the duplicate cultures.

The PT production data of these cultures (Figure 3b) show that the total PT production increased from 1.1 mg/g at 25 mM Na^+ to 2.4 mg/g at 100 mM Na^+, after which the PT production in the suspension dropped slightly to 1.5 mg/g. The PT-concentration in the supernatant increased steadily from 0.15 mg/g to 2.4 mg/g at 150 mM of Na^+, Thus, at salt concentrations below 125 mM, most PT is associated with the cells. The total PT production is quite comparable between 50 and 175 mM Na^+, while the proteolytic activity showed a maximum between 100 and 125 mM Na^+.

The finding that the total PT production in the range of 50 to 175 mM of Na^+ is more or less constant is in conflict with earlier work [16]. The difference in findings can be explained by the fact that the authors established a constant conversion factor from optical density to dry weight, and measured OD for their experimental data. This study showed that the conversion factor of OD to dry weight is not constant when the amount of Na^+ is not constant. Also, the authors measured PT in the supernatant only, which is valid at high salt concentrations when all PT has dissociated from the cells. However, Figure 3b shows that at lower salt concentrations most PT is associated with the cells.

Though further quantification of these results in bioreactor cultures is required, the data presented here show that the total Na^+ concentration should preferably not exceed 75 mM, since protease activity is maximal between 100 and 150 mM Na^+, while above 150 mM, where proteolytic activity is also low, the growth rate is much lower than at 75 mM Na^+. However, all commonly used *B. pertussis* production media contain at least 120 mM Na^+. Since 140 mM Na^+ is physiological for the host organism, it seems curious that the growth rate of *B. pertussis* at this salinity is that low. However, the salinity of the respiratory tract surface liquid, i.e. the target of *B. pertussis*' colonization, only contains 63 mM sodium [20], i.e., a sodium concentration at which *B. pertussis* grows quite rapidly. Apparently *B. pertussis* is quite well adapted to the conditions in the host organism.

The higher growth rates at 75 mM sodium allow for a 30% shorter cultivation time if compared to cultivations at 150 mM. Therefore, using low sodium concentrations would increase the volumetric productivity of the bioreactor. For both cellular and acellular vaccine production, cultivations could be done at low salt concentration. While the cells to be used for a cellular vaccine just need to be washed and inactivated, the cells for an acellular vaccine could be extracted with high sodium concentrations, releasing both PT and FHA [2].

3.4 Fe^{2+} concentration

Since PT causes host cell death and subsequent lysis, releasing Fe-containing compounds, PT production could be seen as a means to acquire iron. Hence, PT expression might be modified by the levels of available Fe. While total absence of iron would cause iron-limited growth, possibly a low Fe concentration, corresponding with the situation in the host organism, could enhance toxin production. This hypothesis was tested in medium containing various amounts of iron, shown in Figure 4.

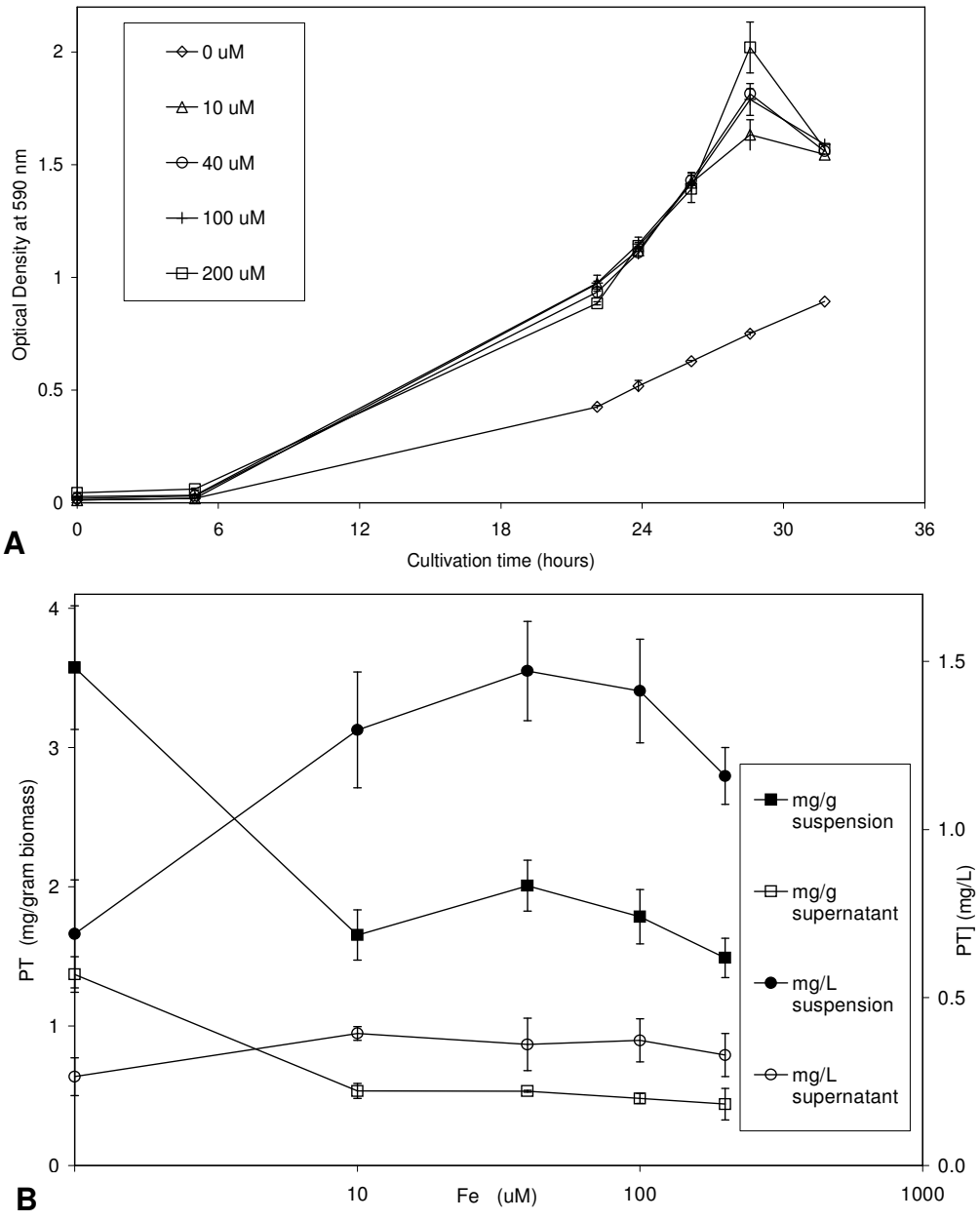

Figure 4. Growth curves (4A) and the production expressed as the mg PT per ml and PT per gram biomass of total and free PT (4B) in media containing 0 to 200 µM Fe. Note that the x-axis is logarithmic so that a value of 1 corresponds to 0 µM. The error bars represent the standard deviation of samples from the duplicate cultures.

The medium that contained no added iron showed a much slower growth than the cultures that contained 10 to 200 µM added iron (Figure 4a). The cultures with no added iron yielded a dry weight of 0.19±0.002 g/L, significantly less than the average dry weight of the 8 other cultures, which was 0.75±0.029 g/L (no data shown). The spent culture medium of the cultures without added iron showed that less than half of the added lactate was used while all glutamate was consumed (no data shown). Apparently Fe^{3+} present as a contaminant and from carry over (<0.5 µM, data not shown) was not enough to fully sustain growth. Although the specific PT production of 3.6 mg/g biomass was quite high (Figure 4b), the total amount of PT produced expressed as mg/L was the lowest of all cultures.

For the cultures containing between 10, 40 and 100 µM Fe, the expression of PT was comparable: 1.79 ±0.18 mg/g biomass. Cultures with an iron concentration of 200 µM produced approximately 35% less than the optimum, which is in agreement with the earlier findings [5]. The proteolytic activity was similar for all cultures (data not shown).

The data presented here show that low [Fe^{3+}] increase specific PT production, but at the same time limit the amount of biomass produced. The low total PT content per unit biomass in the iron-limited cultures is unacceptably low for acellular vaccine production. However, the high specific PT production could be exploited in cellular vaccine production to yield a vaccine with a low biomass content, yet a high PT content. The low biomass, and thus the low LPS content of the vaccine should be less reactogenic in humans.

3.5 Influence of temperature on PT production

A wide range of temperatures is used to cultivate *B. pertussis*, without a clear optimum reported. In order to find an optimum growth temperature for PT production the organism was cultivated between 32.6 and 39 °C. The results of these cultures are shown in Figure 5. At 39°C the *B. pertussis* strain 509 hardly grew, but at all other temperatures the cultures grew to comparable densities with comparable growth rates (Figure 5a). This is to be expected for an organism that infects the respiratory tract, where inhaled air constantly cools the surface of the surrounding tissue somewhat, i.e. the ambient temperature in the respiratory tract is less than the body temperature of the host. Figure 5b shows that PT production was fairly constant between 33 and 36 °C. PT was largely associated with the cell, while at 37 °C the PT production was at least 30% lower. The maximum proteolytic activity coincided with the maximal PT production. At 32.6 °C, the proteolytic activity declined to approximately 70%, while the PT production declined only 20%.

Although the production of PT and therefore other virulence factors is optimal below 36 °C, many producers and researchers cultivate *B. pertussis* at 37°C [12, 14, 21]. While *B. pertussis* grows well at temperatures below body temperature (33 to 36), it does not grow well at temperatures higher than 37 °C. Poor growth at these higher temperatures (38-39), i.e. a situation corresponding with fever of the host, may seem curious, but fever is normally minimal during a pertussis infection.

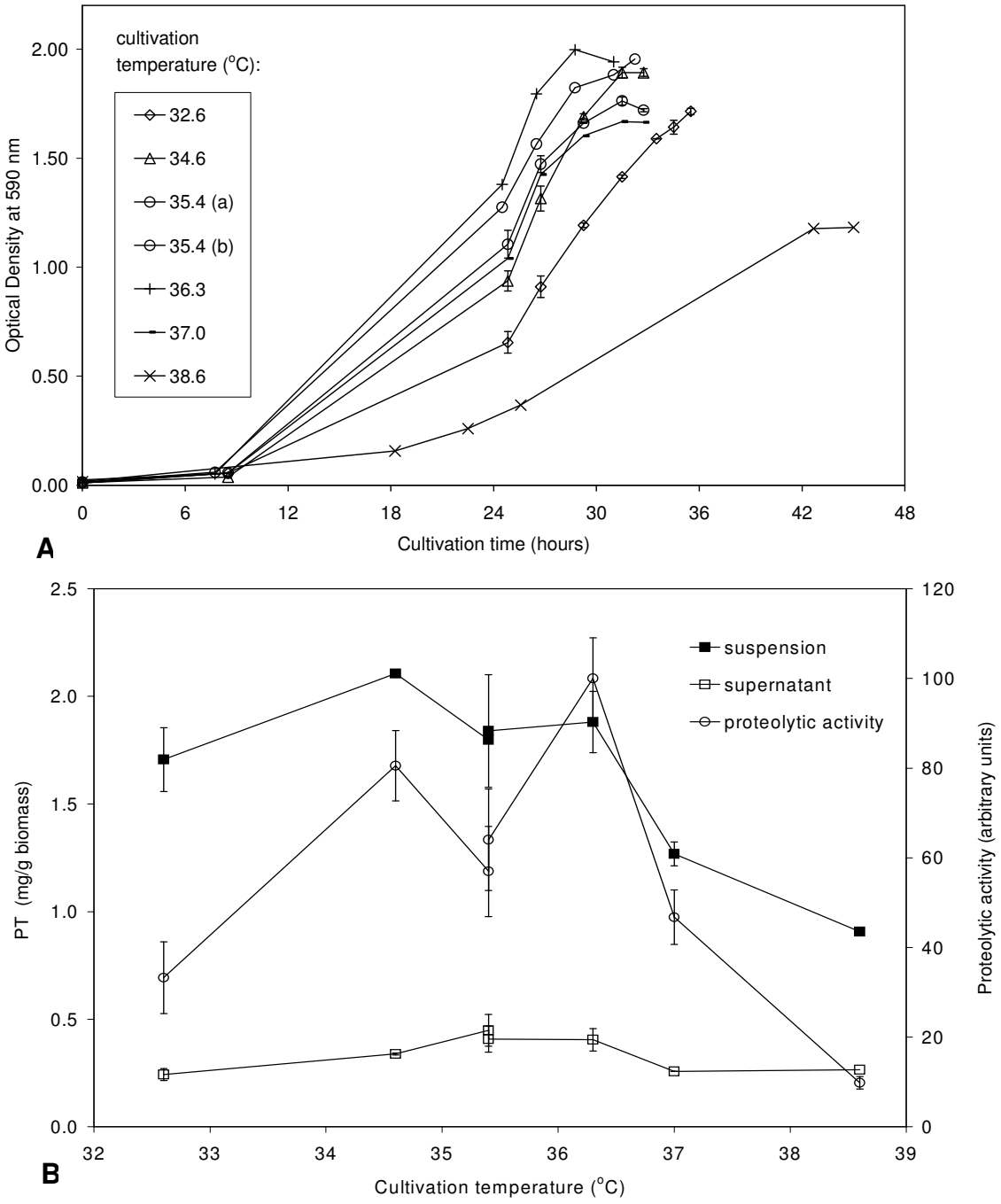

Figure 5. Influence of temperature on the growth curves (5a), the production of PT and proteolytic activity (5b) measured in the suspension at the time of harvest. All cultures were carried out with a temperature variation of less than 0.2 °C. The cultures at 35.4°C were carried out in quadruplicate as control cultures. The error bars represent the standard deviation of samples from the duplicate cultures.

4. Concluding remarks

Using straightforward experimental methods, the present study revealed a number of new aspects of the production of PT, which are relevant for both cellular and acellular vaccine production. Experiments were done in a chemically-defined medium instead of a standard production medium with complex medium components to prevent experimental variation due to the presence of undefined components. However, it stands to reason that the parameters studied here also affect *B. pertussis'* virulence factor production in currently used production media. For example, *B. pertussis* is often grown at 37 ^{o}C, while this study shows that PT production is at least 30% higher around 34 ^{o}C. The magnitude of the effects may differ, but the influence can be expected to be similar to the results in this study.

Monitoring proteolytic activity during the cultivation was shown to be a useful tool to define when to harvest a culture, minimizing degradation losses during the cultivation. Though not studied here, proteolytic activity during the purification procedures may also prove to be useful to prevent degradation losses. Even though proteolytic activity leads to loss of PT-subunit 1, none of the other studies showed degradation of PT, possibly because of the use of ELISA's. Since most of the antibodies in vivo are directed against subunit one, degradation of a fraction of subunit 1 may affect vaccine potency.

At sodium concentrations above 75 mM the growth rate of the organism declined, while the PT production was similar. At higher sodium concentrations most PT was present in the supernatant, which is undesirable for cellular vaccine production. Since most cultivation media for *B. pertussis* contain more than 120 to 150 mM sodium, a considerable gain in PT content of cellular vaccines is achievable. Low sodium concentrations also allow for 30% shorter cultivations when compared to sodium concentrations of currently used production media, which would also benefit acellular vaccine production.

Though varying the iron content did not improve PT production between 10 and 100 µM iron, higher concentrations lowered the PT production. Since the iron content of complex media is not measured, it may be advisable to monitor the iron content of the complex raw materials. At iron limitation, the total PT production was low, but per unit biomass the PT content was quite high. This condition could be used to produce a cellular vaccine with a low biomass content, and corresponding low reactogenicity.

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CHAPTER 4

Improving the cellular pertussis vaccine: increased potency and consistency

Summary

Although Europe, Canada and the US have switched from cellular to acellular pertussis vaccines, most developing countries will continue to use the more cost effective cellular vaccine. Consistency of production however is the typical problem inherent to cellular vaccines. Optimising the production process of cellular pertussis bulk suspensions using product potency as a measure is not possible, since the mandatory animal test to measure potency has little discriminatory power. To circumvent this problem, this study focussed on measuring process parameters related to consistency and potency instead, even though the extent of those relationships could not be quantified. Critical evaluation and modification of individual process steps lead to 2 optimised production processes, NVP-96 and NVP-THIJS. These were compared to the original NVP production process in terms of antigen and biomass content, potency, toxicity and immunogenicity in mice. The batch to batch variation for both optimised products was clearly less than the original product for all parameters tested. The biomass content of the NVP-THIJS product was 15% lower than that of the NVP-96 product, while the immunogenicity in mice was 2 to 3 fold higher. The stability of the NVP-THIJS product remained higher then the NVP-96 product over a period of 2 years, while the decline of the potency of both suspensions was comparable.

key words: cellular pertussis vaccine, consistency, production process

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1. Introduction

The US, Canada and most countries in Europe have switched or will switch from a cellular to an acellular pertussis vaccine. The adverse events associated with a cellular pertussis vaccine are the primary reason for this switch. Though less reactogenic, the efficacy of most acellular vaccines is less than that of a cellular vaccine [1,2]. Acellular vaccines are significantly more expensive to manufacture due to the extensive down stream processing. Also, the amount of *B. pertussis* cells needed to produce one dose of acellular vaccine is sufficient to make 5 to 25 doses of cellular vaccine [2]. Developing countries will therefore not switch to acellular pertussis vaccines in the near future. The Netherlands Vaccine Institute (NVI) [1] undertook the work described in this paper to increase the potency of the product and the consistency of the existing production process in the Netherlands.

The production process of cellular pertussis vaccine suspensions is relatively straightforward. *B. pertussis* is cultivated, centrifuged, resuspended and inactivated (Table 1). To produce these suspensions consistently however, is far more complicated. Historically, the cellular vaccine is known for its large batch to batch variation, as well as for variations between different producers [3]. Since the antigenic composition of the pertussis cells is determined during the cultivation, the reproducibility of this step is crucial. Unfortunately, often poorly defined components like casein-hydrolysates and yeast extract are used in *B. pertussis* media, inherently causing batch to batch variation in the medium and thus in the cultivation step. Therefore the duration of the growth phase and the final cell density can vary significantly from culture to culture. Furthermore, most pertussis vaccine production processes are time-based rather than event-based, meaning that for example the culture is harvested on a fixed culture time independent of the growth phase of the culture. This, in combination with the variability of the growth curves, leads to significant variations in both the quality of the inocula as well as the quality of the harvest of the production runs. Finally, the time between harvest of the living *B. pertussis* cells and their inactivation can vary significantly from producer to producer, or even from batch to batch, if the batch production records allow a wide time window between harvest and inactivation.

Most existing production processes for whole cell pertussis vaccines were registered 4 or more decades ago and are essentially unchanged over this time, mainly because of regulatory hurdles associated with introducing process changes. Consequently, these processes are not up to date with present day

[1] The NVI is the result of a merger in 2003 between the Foundation for the Advancement of Public Health and Environmental Protection (SVM) and Sector Vaccines of the Foundation for the Advancement of Public Health and the Environment (RIVM).

knowledge and techniques. Therefore, during a scale-up program from 350 L to 1000 L volume to produce pertussis vaccine the consistency of the process was improved. The scale-up and improvement of the process consistency was done in two steps, which are summarized in Table 1. In the first step the original 350 L process (NVP) was changed to a 1000 L scale process (NVP-96). Besides the change in scale, changes were made regarding the type of media components used such as the yeast hydrolysate, the medium preparation procedure, the inoculum culture, the cultivation time, control of dissolved oxygen (DO) levels, as well as the storage duration of the cells prior to inactivation. In the second step, further improvements were made resulting in the NVP-THIJS process. Changes included a switch to a chemically defined medium [4], definition of harvest time based on biomass concentration rather than time, as well as a change of preservative and the inactivation procedure.

In this paper the consistency of the three processes, the two new production processes (NVP-96 and NVP-THIJS) and the original NVP process, is compared. A number of off- and on-line measurements was used to compare the consistency of the 3 production processes such as optical density, dry weight, metabolites and pH. The product characteristics of the inactivated bulk suspensions of the NVP-96 and the NVP-THIJS suspensions are compared to the NVP product in terms of dry weight per vaccine dose, Pertussis Toxin (PT) and Filamentous Hemeagglutinin (FHA) content, as well as immunogenicity in mice. Finally, the potency of the inactivated suspensions was tested in the Mouse Potency Test (MPT) [5], which is a test with low discriminatory power but required by regulatory authorities to release the pertussis vaccine. Also, the stability of the inactivated bulk suspensions with respect to potency was assessed using the MPT in a real time stability study over a period of 2 years. Not all parameters were available for the NVP process, so for some parameters only the NVP-96 and NVP-THIJS processes are compared.

Table 1. The differences between the NVP, NVP-96 and NVP-THIJS production processes, using strains 134 and 509. Abbreviations used: T-Temperature, DO-Dissolved Oxygen Tension, IOU-International Opacity Unit.

process step	NVP	NVP-96	NVP-THIJS	parameter altered/investigated
medium raw materials	B2 medium -wet pressed yeast -casamino acids	B2 medium -dried granular yeast -casamino acids	THIJS medium -chemically defined medium	medium quality/reproducibility - wet pressed yeast extract vs. powdered yeast extract - casamino acids batch to batch variation - strain dependent growth on media
medium preparation	-pre-sterilise bioreactor -add 2 heat and 2 filter sterilised stock solutions	-sterilise powder medium *in situ* -add 1 filter sterilised stock solution	-sterilise powder medium *in situ* -add 1 filter sterilised stock solution	filter sterilisation in combination with heat sterilisation versus heat sterilisation of individual components
pre-culture	-ampoule to 200 ml shake flask (48-72 hr. cultivation in Verwey medium)	-ampoule to 200 ml shake flask 1, shake flask 1 to 200 ml shake flask 2 (48-72 hr. + 24 hr. cultivation in Verwey medium)	-70°C culture to 200 ml shake flask (24 hr. cultivation in THIJS medium)	reproducibility of the pre-culture in terms of lag-phase and final OD
inoculum culture (24 hrs)	-200 ml shake flask to 800 ml shake flask	-200 ml shake flask 2 to 16 L bioreactor	-200 ml shake flask to 16 L bioreactor	reproducibility of the inoculum-culture in terms of lag-phase
production culture **(control of T and RPM)**	-350 L culture -no control of DO -cultivation time: strain 134: 72 hours strain 509: 72 hours	-1000 L culture -control of DO -cultivation time: strain 134: 48 hours strain 509: 72 hours	-1000 L culture -control of DO -cultivation time: strain 134 to density 26 ±2 IOU strain 509 to density 26 ±2 IOU	time-based versus OD based harvest
harvest (continuous centrifugation)	-measure IOU directly after harvest	-measure IOU 7 days after harvest	-measure IOU directly after harvest -cool to < 18°C before harvest	minimize impact of harvest procedure on the cells
inactivation & detoxification **(10 min. 56°C)**	-inactivate both strains together -12 weeks storage -preservative: hyamine -pH of inactivation not specified	-inactivate each strain separately -6 weeks storage -preservative: hyamine -pH of inactivation not specified	-inactivation each strain separately -1 night storage -preservative: formaldehyde -pH of inactivation 7.7±0.1	inactivation after >6 weeks storage period versus direct processing in 10 mM formaldehyde

2.0 Materials and Methods

Media. The B2 medium [6] and the Thalen-IJssel (THIJS) medium [4] were used. The yeast extract used in the B2 medium is derived from yeast cells, either wet-pressed paste or dried and granulated yeast, which are suspended in saline and autoclaved at 110 °C in order to lyse the cells. The resulting suspension is clarified, filter sterilised and stored at 4°C until use. Composition of THIJS basal medium in g l^{-1}: NaCl, 3.319; NH_4Cl, 0.107; KH_2PO_4, 0.5; KCl, 0.5; $MgCl_2.6H_2O$, 0.1; TRIS, 1.525; Na-glutamate.H_2O, 1,87; L-lactate (40 % weight volume^{-1} %), 3.76 ml; 5 M NaOH solution, 2.071 ml. Supplement composition in g l^{-1}: L-cystine, 4.0; $CaCl_2$.$2H_2O$, 2.6; glutathione (reduced), 10.0; $FeSO_4$.$7H_2O$,1.0; nicotinic acid, 0.4; L-ascorbic acid, 2.0 and 1 M HCl, 120 ml. The basal medium was heat-sterilised at 121℃, the supplement was filter sterilised directly into the bioreactor just before inoculation. Per litre basal medium, 10 ml supplement was added.
Strains. Strain 134 and 509, which are both used in DPTPolio vaccine applied in the National Vaccination Program (NVP) in the Netherlands at 8 IOU per dose each, were used in all experiments. A lyophilised ampoule of the NVI working seed lot was used to inoculate 200 ml Verwey medium [7] on a 200 RPM rotary shaker at 35℃ for 2 to 3 days until it reached 20±4 IOU. Of this culture, 10 ml were used to inoculate a second shake flask with 200 ml Verwey medium. After incubation for 24 hours at 35℃, glycerol (87% weight volume^{-1}) was added to a final concentration of 10% volume volume^{-1}, after which the suspension was frozen in aliquots of 10 ml at -70℃. The preculture medium was inoculated with 10 ml of these -70℃ working seed lots. The same procedure was carried out for the THIJS medium, with the difference that the preculture medium consisted of THIJS medium as well.
Cultivation, harvest and inactivation equipment. The 350 L bioreactor for the NVP cultivations was constructed by Contact Flow, currently named Applikon Dependable Instruments (ADI, Schiedam, the Netherlands), and controlled by an analogue measurement and control system, the Bilthoven Unit, produced to RIVM specifications by ADI. The 16 (BIO24) and 1000 L (BIO1200) bioreactors, the 160 L inactivation vessel (BIV200) as well as the corresponding service units and ADI1050 controller were also built by ADI. The measurement and control system supervising the ADI1050 was a HP75 Unix machine running the Man-Machine-Interface designed to RIVM specifications by Compex (Compex, Alphen, the Netherlands). The continuous centrifuge Westfalia Separator CSA 8 was used to harvest the cells at the end of the cultivation (Westfalia A.G., Oelde, Germany).
Biomass determinations. The optical density of the suspensions was measured on a Vitalab 10 (Vital Scientific, Dieren, the Netherlands), at 590 nm. One optical density unit corresponds to 20 International Opacity Units (IOU), which in turn corresponds to approximately 0.5 g l^{-1} dry weight. Dry-weight determinations were done by centrifugation at 8000 g for 10 minutes of 40 ml of culture. After drying the cell pellets for 24 hours at 80℃ the cell pellets contained less than 0.5% water as

determined by titration with iodine in a Mitsubishi moisture analyser (Mitsubishi, Tokyo, Japan).

Metabolite determinations. Glutamate was determined using an HPLC method as described before [8]. Lactate was determined with a YSI 2700 glucose and lactate analyser (Yellow Springs Instruments Co., Yellow Springs, USA).

Intracerebral Mouse Protection Test. Briefly, groups of NIH/RIVM out bred mice (n = 16) weighing 10-14 g. were immunised intraperitoneally (i.p.) with a dilution range corresponding with 5 - 1- 0.2 - 0.04 OU, of the whole cell suspensions (WCS) under test, the in-house reference preparation Kh 96/1 is calibrated against the international standard IS 94/532 and contains 11.8 IU/ml and is also immunized in 5-fold dilution steps. The number of dead mice was recorded daily until day 28. For potency calculation, only mice dying from day 17 to 28 were taken in account. Based on the percentage of surviving mice per vaccine dilution, the potency of vaccines is estimated by means of probit analysis.

Mouse Weight Gain Test. The MWG-test was carried out according Ph. Eur., Monograph 2005:0161, (2.7.7). Groups of ten NIH/RIVM out bred mice (14-17 g.) were vaccinated i.p. with a volume containing a half-human dose of Whole Cell Suspension (WCS) under test, i.e. 8 OU, and in-house reference, respectively. The control group received an equal volume of sterile saline. Animals were observed for 7 days and body weight was recorded after 16 hours, 3 and 7 days. Vaccines were considered non-toxic when passing the EP-requirements: a) the total weight of the mice from the vaccine group 3 days after treatment was the same or higher than the initial weight, b) at the end of seven days the average weight gain of the vaccine group was not less than 60% of the control group and c) not more than 5% of the animals died during the test period. Additionally, mice were bled after 28 days to determine the immunogenicity of the WCS under test by means of serology.

Immunogenicity assays. Immunogenicity of the WCS was assessed by determining the sera from MWG-test for antibodies against *B. pertussis* specific antigens such as PT, FHA, pertactin and fimbriae 3 in ELISA. IgG antibodies against the *B. pertussis* surface antigens were measured in an ELISA, using a suspension of the international challenge strain 18323 to coat the 96-well plates [9]. IgG antibodies in the MWG sera against PT, FHA, pertactin and fimbriae 3 were measured in an indirect ELISA, pre-coating the plates with purified antigen (±1 µg/ml.) The coated plates were incubated with serial dilutions of MWG-sera, bound IgG-antibodies were detected using goat anti mouse IgG-biotin/streptavidin-PO system (Amersham, U.K.) and visualised with a TMB-substrate solution. The antibody concentrations were calculated by means of a 4-parameter fit. The individual antibody titers of each mouse were log-transformed, and thus averaged per group of 10 mice. These log-transformed averages were used to compare the immunogenicity of the NVP-96 and the THIJS products.

3. Results

First, the different factors affecting process consistency and the changes made in these factors are discussed, as well as the rationale behind the choices made. Next, the three processes NVP, NVP-96 and NVP-THIJS are compared in terms of biomass, virulence factors and LPS content per dose. Finally, bulk suspensions are compared using the Mouse Potency Test (MPT), mouse weight gain test (MWG), antibody response and the stability of the potency in time.

3.1 Factors affecting process consistency

3.1.1. Medium raw materials. Since the wet-pressed bakers yeast that is used to prepare yeast-extract for B2 medium is metabolically active, the composition is likely to change during its limited shelf life. In contrast dried yeast has a shelf life of at least 6 months and is metabolically inactive, i.e. stable in composition. Therefore, in the first improvement step (NVP to NVP-96) wet-pressed yeast was replaced with dried yeast as raw material for yeast extract preparation. Yeast extract was in house prepared, since using commercial powdered yeast extract yielded lower growth rates and lower cell densities (no data shown).

The casamino acids in the B2 medium, which are derived from acid-hydrolysed casein, inherently differ in amino acid composition from batch to batch. The impact this varying composition has on the growth of *B. pertussis* strain 134 in shake bottles, is shown for various casamino acids lots in Figure 1a. Also, strain 509 and strain 134 show large differences in growth curves on the same batch of casamino acids, as shown in Figure 1b. Strain 509 can produce large amounts of poly-hydroxy-butyrate during cultivations on B2 medium [10]. This difference in growth and metabolism could be caused by a difference in outer-membrane structure between the strains: strain 134 LPS lacks the terminal tri-saccharide present in 509 LPS [11], which is likely to affect membrane permeability. Thus, not only do *B. pertussis* cultivations depend on the batch of casamino acids used, but also on the strain to be cultivated. The use of poorly defined components, like casamino acids and yeast extract, in the B2 medium inherently leads to variation in medium quality. However, the amount of variation in the NVP-96 process could be limited by purchasing the undefined components from the same supplier and testing lots on a small scale before use in routine production.

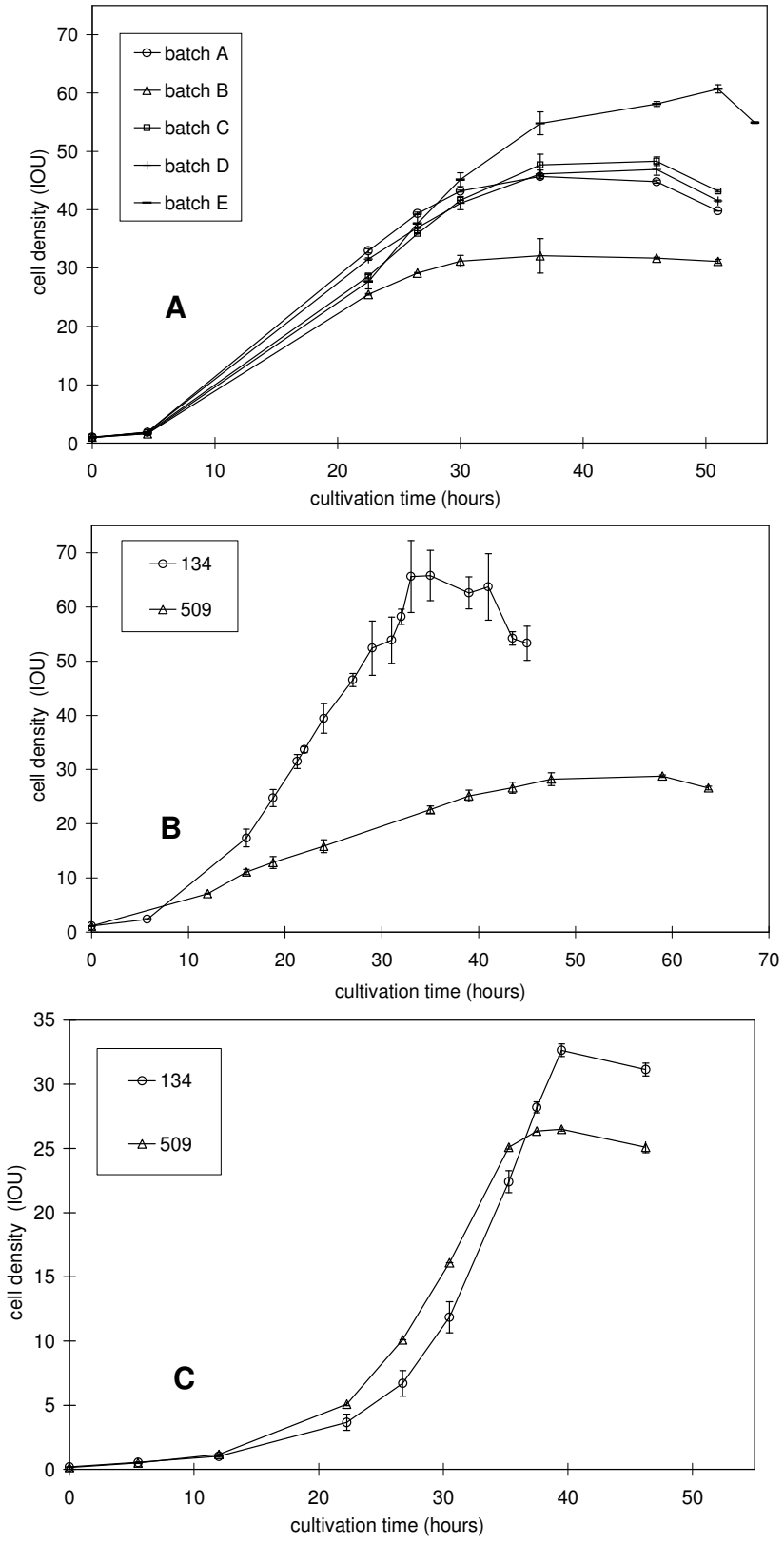


Figure 1. Typical growth curves of strain 134 on 5 different lots of casamino acids (A), of strain 134 and 509 on one lot of casamino acids (B), and growth of strain 134 and 509 on THIJS medium (C). Cultivations were carried out in duplicate; the standard deviation is indicated with error bars.

In the second improvement step from the NVP-96 to the NVP-THIJS process the problems associated with the use of undefined components were completely solved by using a chemically defined medium. The chemically defined Stainer-Scholte medium [12] was modified by Thalen [10], resulting in the balanced THIJS medium, which was later optimised for PT production [6]. In this medium both strain 134 and 509 grew to approximately the same density with similar growth rates, shown in Figure 1c. The final density (Figure 1c) is lower than on the B2-medium (Figure 1b), because the initial substrate concentrations were deliberately chosen low in order to avoid waste metabolism [10]. Thus, the consistency of the cultivation on THIJS-medium is markedly increased over the NVP-96 medium.

3.1.2. Medium preparation. The NVP process employed stock solutions to prepare the production medium, which was practical for 350 L but not for 1000 L scale. Typically one batch of yeast extract was used for multiple production runs. Since the shelf life of complex media is limited, the use of stock solutions was avoided for the 1000 L scale. Medium was prepared per batch rather than for a number of batches. The components that could be heat sterilised such as the salts, glutamate, casamino acids, and starch were delivered into the 1000 L bioreactor in powdered form, and sterilised *in situ*, at pH 5.8±0.2 to reduce degradation of the amino acids by Maillard reactions. Since yeast extract contains a number of heat sensitive vitamins, it was added to the production medium as a filter-sterilised solution, together with glutathione, $FeSO_4$, and $CuSO_4$, just prior to inoculation. For the NVP-THIJS process the basal medium components of the THIJS medium such as the salts and substrates were delivered as powder and sterilised in the bioreactor, while the supplement was prepared and added to the bioreactor as a filter-sterilised solution, just prior to inoculation.

3.1.3. Pre and inoculum culture. In all three processes the procedure to prepare the inoculum for the large scale production bioreactor consists of a preculture, which is inoculated from a working cell bank. This culture is used to inoculate the inoculum bioreactor, which is in turn used to inoculate the production bioreactor. Both the NVP and the NVP-96 process used lyophilised ampoules to inoculate the pre-culture medium into a shake flask. In the NVP process this shake flask was directly used to inoculate the inoculum culture. However, the lag phase of the cultures inoculated with an ampoule is rather variable (no data shown), which resulted in OD variations in the inoculum culture and thus in the production culture as well. In the NVP-96 process the biomass of the first shake flask was used to inoculate a second shake flask, which yielded a more reproducible pre-culture, and thus also more reproducible inoculum and production cultures. To further shorten and standardize the preculture phase, glycerol stocks were prepared for the NVP-THIJS process by dividing the second shake flask in 10 ml aliquots in the presence of 10% glycerol, which were stored at -70°C. These glycerol stocks were then used to inoculate the 200 ml NVP-THIJS preculture.

Using glycerol stocks instead of lyophilised ampoules reduces the lag time, shortening the total duration of the pre-culture to 24 hours.

For the NVP process the 350 L bioreactor was inoculated with 800 ml of pre-culture, which corresponds to an inoculum of 0.2 %. The disadvantage of using such a small inoculum is that the quality of the inoculum culture strongly influences the lag phase in the production culture, and thus the total cultivation time. Since *B. pertussis* cultivations were harvested based on cultivation time rather than growth phase, the suspension harvested may not always have been from the exact same growth phase. In order to reduce lag periods and to monitor and control the inoculum culture better, a computer-controlled 16 L bioreactor was introduced in the NVP-96 and NVP-THIJS process, resulting in a 1.6% volume inoculum and a short lag-period in the production culture.
3.1.4. Implementation of Dissolved Oxygen control (DO). In the 350 L bioreactor of the NVP process the dissolved oxygen concentration (DO) was not controlled and consequently varied during the run due to the increasing biomass concentration. However, the oxygen transfer rate (OTR) into the bioreactor by headspace aeration alone was sufficiently high to keep the DO from reaching 0% saturation. The OTR of the 1000 L bioreactor in the NVP-96 and NVP-THIJS process was, however, considerably lower than that of the 350 L bioreactor (no data shown), which could lead to oxygen limitation and large variations in growth. Therefore to prevent oxygen limitation, DO-control was implemented in the 1000 L bioreactor. Control was accomplished by sparging oxygen into the liquid phase of the bioreactor. An additional advantage is that the control unit of the 1000 L bioreactor besides monitoring and controlling the DO also monitors the dissolved-oxygen-controller-output (DOCO), a number which corresponds with the quantity of oxygen through the sparger into the bioreactor [13]. Since the DOCO corresponds with the oxygen consumption by the culture, it is possible to use the DOCO as a parameter to monitor growth on-line. For the NVP-96 process, the DOCO profiles and the corresponding optical densities of 2 cultures of strain 134 are shown in Figure 2a, as well as for strain 509 in Figure 2b. As apparent from the OD values in Figure 2, strain 134 grew significantly faster and to a higher cell density than strain 509. The difference in growth rate was also reflected in the DOCO pattern: the DOCO's of strain 134 rose more steeply than the DOCO of strain 509, and remained higher for most of the cultivation. The DOCO patterns per strain, however, were quite reproducible.

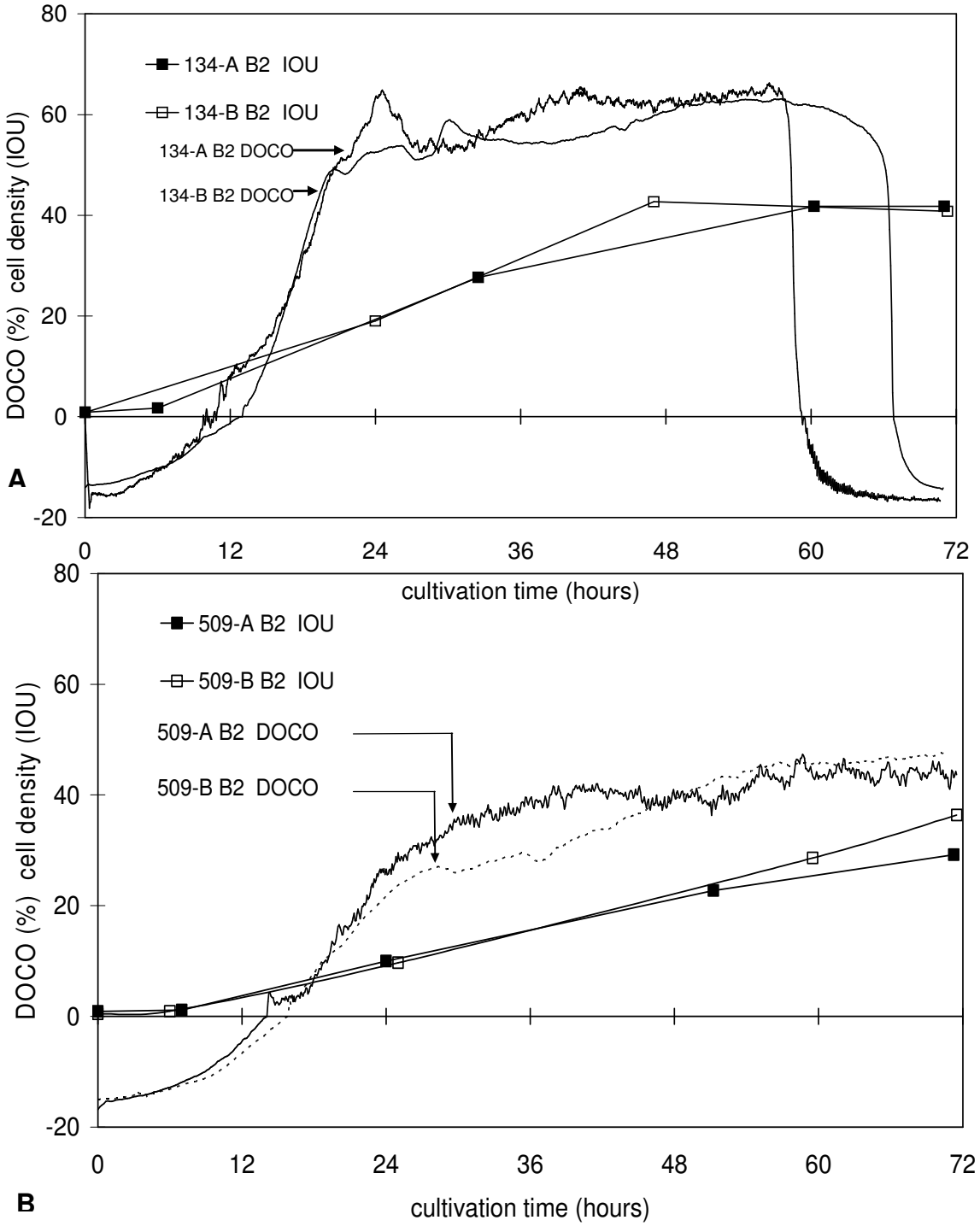


Figure 2. Cell densities for 2 NVP-96 cultivations per strain (A and B) on B2 medium for strain 134 (A) and 509 (B) as well as the corresponding dissolved oxygen controller output (DOCO) signal.

3.1.5. Introduction of an event-based harvest criterion. The NVP production process was strictly time based, meaning the harvest was started after a given amount of culture time, without taking the batch to batch variation in growth rate and lag time into account. Also the difference in the culture duration between strain 134 and 509 was not taken into account, i.e. both strains were harvested after 72 hours (Table 1). Since there is a direct link between the DOCO and metabolic activity, the DOCO data can be used to determine a harvest criterion. An example of the relevance of the DOCO parameter in relation to the time of harvest of the cultures is shown in Figure 2 for the NVP-96 process. The DOCO of batch 134-A suddenly decreased after 60 hours of growth, probably reflecting the point where all available substrates in the complex B2 medium were consumed (Figure 2a). A similar pattern was observed for culture 134-B, only after 70 hours of cultivation (Figure 2a). When these cultures were harvested at 72 hours, which is well after the decrease of the DOCO signal, DNA was released (no data shown), resulting in a viscous slurry that could not be resuspended homogeneously. For strain 509 (Figure 2b) the decrease in DOCO did not occur before 72 hours and DNA release did not occur. The DNA release was only observed for strain 134, not for strain 509, suggesting that a drop in the DOCO signal, leads to lysis of the cells.

No DOCO data are available for the 350 L NVP cultivations, but the fact that severe cell-lysis was never observed during harvest or inactivation of 350 L suspensions suggests that complete consumption of substrates did not occur under NVP conditions for strain 134. The difference in the time at which substrate depletion occurs can be explained form the difference in inoculum size between the NVP and NVP-96 process. The use of a larger inoculum for the NVP-96 process (1.6%) versus the NVP process (0.2%) means that the NVP-96 culture will be approximately 3 population doublings shorter than the NVP culture. Assuming a doubling time of about 5 hours, this means that substrates will be depleted 15 hours earlier than for the NVP culture. Therefore, in order to avoid the drop in oxygen consumption and the subsequent lysis of cells during further processing, all subsequent 1000 L NVP-96 cultures of strain 134 were harvested 24 hours earlier than the NVP cultures, i.e. after 48 hours of cultivation. The drop in DOCO signal was consequently never observed in any of the subsequent production cultures (no data shown).

3.1.6. Chemically defined THIJS medium and DOCO-data to establish a harvest criterion. Since THIJS medium contains only lactate and glutamate as substrates, the depletion of these substrates could be linked to a drop in oxygen demand. In Figure 3, the point at which lactate is consumed exactly coincides with a sudden drop in oxygen demand (Figure 3, solid arrows), while there was still glutamate present. Adaptation to growth on glutamate only can be expected to take some time, explaining the drop and subsequent rise in oxygen demand. Several hours

later in the cultivation, glutamate was also completely consumed, corresponding to the second drop in oxygen demand (Figure 3, dashed arrows), after which the oxygen flow through the sparger stopped completely. The results in Figure 3 allow to establish a direct link between the DOCO signal and the subsequent depletion of the 2 substrates. This cannot be done for the DOCO signals in Figure 2, since these culture is grown on complex B2 medium which contains numerous substrates. On the basis of the oxygen consumption pattern and the increase of the optical density, the culture should be harvested when the OD reaches 25 IOU, i.e. well before substrates are depleted, in order to prevent cell-lysis. Since continuous centrifugation took approximately 6 hours, the culture was cooled rapidly to below 18°C during harvest in order to stop the culture from depleting the substrates and thus avoiding the risk of lysis and subsequent DNA release.

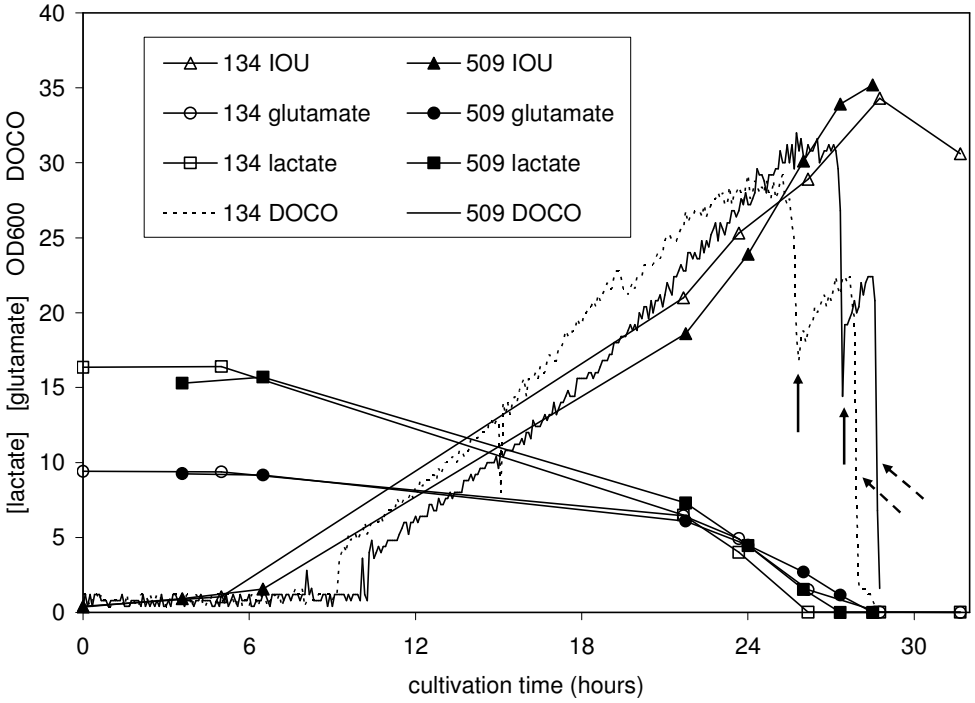


Figure 3. Optical density, substrate concentrations, and DOCO of cultures with strain 134 and 509 on THIJS medium. The arrows indicate the link between lactate and glutamate depletion and the DOCO signals, more elaborately discussed in the text. The inoculum was 1.6% with a 24 hour culture, i.e. comparable to the inoculum of the 1000 L bioreactor.

All production steps of the NVP-THIJS process are well defined, and allow for little variation in operating procedures. Medium preparation, harvest, and inactivation are not dependent on biological activity so that these steps are all largely automated, and consequently quite reproducible. Since the production culture largely determines the product quality, the process parameters of this step can be used to correlate cultivation data with the potency of the resulting product. The DOCO proved to be useful to monitor the oxygen consumption, and as a fingerprint of the cultivation. As more data are gathered, the hypothesis that similar DOCO profiles always yield a potent product can be evaluated.

3.1.7. Harvest, inactivation and detoxification. The heat inactivation of harvested pertussis suspensions serves to kill *B. pertussis* cells and to inactivate *B. pertussis'* intracellular dermo-necrotic toxin. In the NVP process the harvest of a 134 and a 509 cultivation were mixed prior to inactivation in order to obtain a suspension that could be directly formulated together with the other DPTPolio components. After the harvest by continuous centrifugation, the individual suspensions of strain 134 and 509 were stored at least 12 weeks at 4 ^{o}C prior to mixing and inactivating the strains. Heating at 56 ^{o}C for 10 minutes in the presence of the inactivating agent hyamine killed any remaining viable cells. For the NVP-96 process harvests from a 1000 L cultivation were inactivated per strain separately due to quantity of the harvest. Therefore the storage period between harvest and inactivation could be shortened to one week. However, the 509 suspensions, which in the NVP process were inactivated after 12 weeks of storage, were not always completely inactivated by the inactivation procedure (no data shown), when storage was reduced to one week. Small-scale inactivation studies showed that suspensions should be stored at least 6 weeks to inactivate all cells by heat inactivation. Therefore, the storage period was reduced from 12 weeks for the NVP process to 6 weeks for the NVP-96 process.

However, despite the fact that the metabolic activity of *B. pertussis* cells at 4 ^{o}C is probably very low, even at low metabolic rates a storage period of 6 weeks could alter the characteristics of the suspension. In addition, if the heat sensitive proteolytic activity described by Cyr *et al*. [14] is present, a storage period of 6 weeks could influence the immunogenicity of the suspension. In order to avoid all this, ideally suspensions should be inactivated shortly after the harvest. Since hyamine was not always effective in killing fresh 509 suspensions, a different inactivating agent was needed, compatible with the standard DPTPolio vaccine formulation. Since formaldehyde is present at 0.83 mM in the final DPTPolio vaccine, it was a logical choice to replace hyamine by formaldehyde prior to the inactivation of the NVP-THIJS bulk suspension. The action of formaldehyde is based on the formation of covalent bonds between formaldehyde and free ε-amino groups of amino-acid residues. Thus proteins and peptidoglycan are effectively cross-linked, interfering with biological functions. Because this reaction is pH dependent, [15] the pH of the bulk suspension was raised to 7.8 prior to inactivation. After the inactivation the pH dropped to approximately 7.2 ±0.2, presumably due to the H^{+} formed in the reaction of formaldehyde with ε-amino groups. A concentration of 10 -16 mM per 200 IOU suspension was found to be optimal (no data shown). Approximately 10 mM formaldehyde was consumed in the reaction with cell suspension (no data shown), so lower concentrations might not completely inactivate the suspension. Higher concentrations formaldehyde appeared to lower the MPT (no data shown), which is in good agreement with other studies [16]. Small-scale inactivation experiments were confirmed on a production scale showing that in samples taken immediately after inactivation no

living bacteria could be detected. Since harvesting a 1000 L cultivation took approximately one day, the inactivation was done the next day, leaving only one night of storage at 4 °C.

3.2 Comparison of inactivated bulk suspensions in terms of biomass, virulence factors and LPS per vaccine dose

Since the virulence factors Pertussis Toxin (PT) and Filamentous Heamagglutinin (FHA) are important virulence factors present in almost all acellular vaccines, these were measured using an indirect Elisa for PT and FHA [17]. The indirect Elisa results are difficult to link to exact antigen contents due to the fact that virulence factors attached to the cells behave different from the same antigen in free form. However, the method is quite reproducible and can be used to quantify the variation in content per product. As such, the PT and FHA contents of the suspensions prepared by the 3 production processes should not be compared in absolute amounts, although the variation in the values between the 3 production processes can be compared. For both FHA and PT the variation in the outcome is much higher for the NVP process than for the NVP-96 and NVP-THIJS process, demonstrating the process has become more consistent. The PT-ELISA showed that the NVP-THIJS products had a comparable or higher PT content than the NVP-96 and NVP suspensions. For FHA, the content was comparable for the three processes. However, the variation for the NVP suspension was very high making any difference difficult to observe. Furthermore, since the ELISA only measures exposed antigens, i.e. those that are accessible to monoclonal antibodies, the measured amounts might be underestimated, and thus the differences between suspensions as well. For PT also an enzymatic method is available nowadays [14]. However, the method was not available for the NVP process, while the formaldehyde in the NVP-THIJS bulk suspensions interfered with this PT assay (no data shown). Therefore only the ELISA data are shown (Table 2).

The amount of biomass included in a human dose is based on OD values (opacity units) of the bulk suspension. The Dutch vaccine contained both strains in an equal amount of 8 opacity units. Thus, if the amount of dry weight per OD unit varies, also the dry weight per human dose will vary, and therefore probably also the LPS content. The variation in dry weight for the NVP suspensions was somewhat greater than for the NVP-96 and NVP-THIJS products confirming the better consistency of the latter two processes. The NVP-96 and NVP-THIJS products both showed that they contained less biomass per human dose for the strain 134 than for strain 509, meaning the amount of dry weight per OD unit is lower for strain 134. Under the electron microscope, strain 134 cells were coccoid in shape, while strain 509 cells were more rod-shaped (no data shown), which may account for the difference in optical characteristics between the

strains, and hence the difference in biomass content per dose. For both strains, the NVP-THIJS products contained approximately 15% less biomass per human dose per strain than the NVP-96 products. Apparently the optical characteristics of the THIJS-suspensions differed from the NVP-96 products. Since the dry weight and the LPS content are related, the LPS content of the NVP-THIJS products was also less, approximately 10% lower than that of the NVP-96 products. This clearly shows that optical density of a bulk suspension is a poor way to standardise the biomass content of pertussis vaccine. These results are in line with Csizer *et al.*, [3] who showed that at the same optical density, the biomass content measured as total nitrogen or dry weight can vary up to 40% from batch to batch, and even more from producer to producer. Therefore, the dry weight content of a suspension is a much more suitable parameter to standardize biomass concentration per vaccine dose than OD, not just from batch to batch, but from producer to producer as well.

Table 2. Comparison of bulk suspensions in *in vitro* assays expressed as content per human dose, as if the vaccine only contains one strain, shown with the standard deviation of the mean. In reality the Dutch vaccine contains both strains in an equal amount of 8 opacity units.

production process	**n =**	**content in µg per human dose**							
		PT ELISA		**FHA ELISA**		**dry weight**		**LPS**	
measurement variation		**<40**	**%**	**<40**	**%**	**<5**	**%**	**<10**	**%**
NVP (134 + 509)	5	0.16	±81%	2.6	±396%	356	±9%	n.a.	
NVP-96									
134	5	0.30	±30%	6.6	±46%	399	±7%	6.7	±7%
509	6	0.13	±23%	7.5	±21%	431	±3%	8.8	±4%
NVP-THIJS									
134	3	0.52	±37%	4.8	±42%	346	±5%	5.9	±2%
509	3	0.36	±44%	7.1	±7%	379	±4%	8.0	±11%

Although the NVP-96 and NVP-THIJS products appeared similar in terms of antigen content per vaccine dose, the total biomass and LPS content was less for the NVP-THIJS products, i.e. per unit biomass the NVP-THIJS products contained more antigen. Though it is outside the scope of this study, it is important to realise that a 16 IOU NVP-THIJS vaccine contains less LPS than a 16 IOU NVP-96 vaccine, possibly causing less adverse reactions. However, currently no animal models are available to quantify the relation between the LPS content and adverse events like fever.

3.3 Comparison of bulk suspensions in the MPT and the Mouse Weight Gain test

Despite the fact that the MPT is quite an inaccurate test, it is the mandatory test for the release of whole cell pertussis containing vaccines (EP test 2.7.7). Therefore, also in this study, it is the most important test by which to compare the NVP, NVP-96 and NVP-THIJS production processes. Due to the inherent poor reproducibility of the MPT, the variability of the test itself was evaluated before using it to compare the 3 types of suspensions. By comparing the MPT values after testing the same batch repeatedly, the test-variation was calculated (consistency of testing). This variation was then compared to the batch-to-batch variation (consistency of production) of the various suspensions. The obtained consistency in testing and the consistency of production are shown in Table 3. It should be noted again that the NVP suspensions differ from the NVP-96 and NVP-THIJS suspensions in the sense that the NVP suspensions contain both strain 134 and 509 combined in one suspension, while NVP-96 and NVP-THIJS suspensions contain only one strain per suspension.

Table 3. Consistency in testing, i.e. repeated testing of the same lot, and production of NVP and NVP-96 bulk suspensions in the MPT, measured within 3 months after production. The number of batches used for the evaluation of the consistency of testing is identical to the number of batches used to determine the consistency of production. Note that the variation coefficients pertain to the logarithmic values of the test.

consistency of testing **process:**	**NVP**	**NVP-96**		**NVP-THIJS**	
strain:	**(134 + 509)**	**134**	**509**	**134**	**509**
variation coefficient	18%	19%	15%	17%	16%
number of tests per batch	2 to 4	2	2	2	2
number of batches	7	8	10	3	3
consistency of production					
average MPT (IU/ml)	23	51	51	84	101
95% lower limit	20	38	39	55	69
95% upper limit	27	70	68	130	149
variation coefficient	23%	11%	13%	17%	16%
number of batches	18	5	6	3	3

Table 3 shows that the consistency of testing of the MPT expressed as the variation coefficient of the potency was similar for the NVP, NVP-96 and NVP-THIJS products. Since the consistency of testing apparently had not changed over time, the batch to batch variation of the 3 types of suspensions or the consistency of production were compared, expressed as the variation coefficient in Table 3. The fact that the variation coefficient for bulk suspensions prepared by the NVP-96 and NVP-THIJS processes was lower than or comparable to the

variation coefficient of the test itself, indicates that the MPT cannot discriminate between bulk suspensions produced by either the NVP-96 or the NVP-THIJS process. The variation coefficient of the NVP products was clearly higher than the variation coefficient of the test, which means that based on the MPT not all NVP products are identical, confirming again that the consistency of production of the NVP-96 and NVP-THIJS products is higher than that of the NVP product. Furthermore, the potency of the NVP-THIJS process is higher than that of the NVP-96 process, which is in turn higher than that of the NVP process.

The Mouse Weight Gain test is a mandatory test to measure the toxicity of pertussis suspensions. The NVP, NVP-96 and NVP-THIJS suspensions all passed the EP and NVI toxicity criteria. In recent years the sera from these mice were collected one month after vaccination with the NVP-96 and NVP-THIJS bulk suspensions. For the NVP products sera were not collected at the time. The antibody levels against PT, FHA, PRN, Fim 3 and intact *B. pertussis* cells were used to compare the immunogenicity of the NVP-96 and NVP-THIJS bulk suspensions. The results in Table 4 show that the strain 134 and 509 of the NVP-THIJS bulk suspensions elicited higher antibody responses in mice than NVP-96 suspensions, both against the individual virulence factors, as well as against *B. pertussis* cells. Except for FHA, the antibody response elicited by strain 134 suspensions is approximately 3-fold higher for NVP-THIJS suspensions than for NVP-96 suspensions. For strain 509, the response against the individual virulence factors increased two-fold. The results are in good agreement with the higher potency observed in the MPT for the NVP-THIJS product.

Table 4. Comparison of immunogenicity between the NVP-96 and NVP-THIJS products. For the NVP-96 products 48 - 50 individual sera were analysed per strain, using 5 batches per strain, while for the NVP-THIJS products 29 to 30 individual sera were analysed, using 3 batches per strain. The sera originated from 3 separate animal experiments.

strain 134 antigens	**ratio NVP-THIJS / NVP-96**	**p-value (t-test)**	**strain 509 antigens**	**ratio NVP-THIJS / NVP-96**	**p-value (t-test)**
PT	3.3	0.012	**PT**	2.7	0.037
FHA	1.7	0.136 [1])	**FHA**	1.9	0.057 [1])
PRN	2.8	0.003	**PRN**	1.9	0.045
Fim 3	4.2	< 0.001	**Fim 3**	1.9	0.066 [1])
***B. pert.* cells**	2.7	< 0.001	***B. pert.* cells**	1.8	0.026

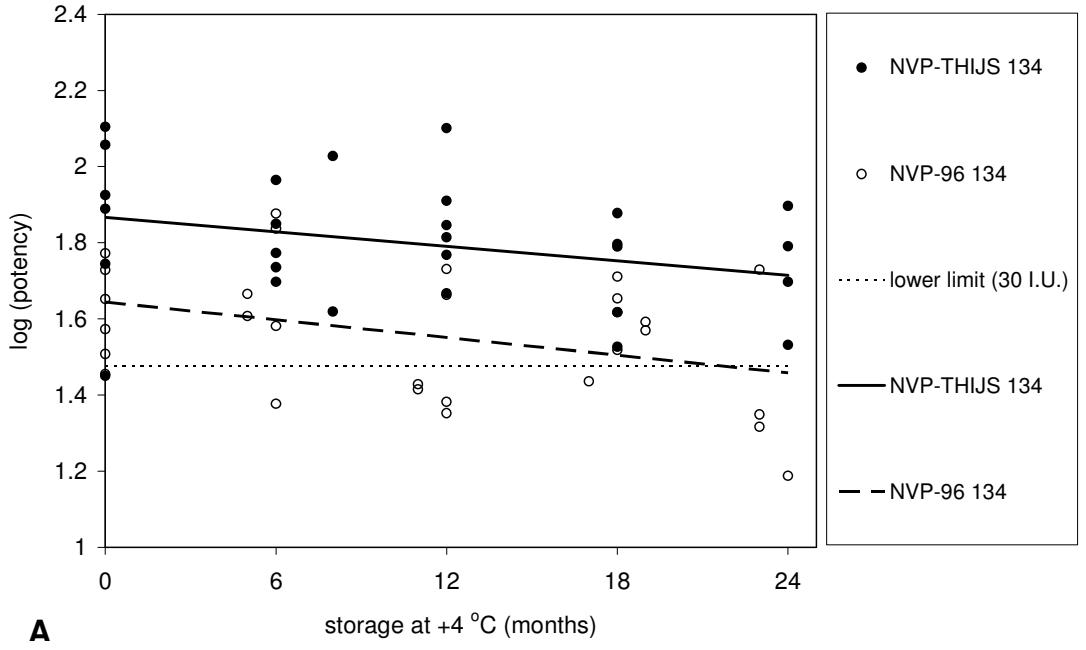
[1]) difference not statistically significant

3.4 Real time stability of NVP-96 and NVP-THIJS suspensions

The data presented so far came from suspensions that were less than 3 months old, so a real time stability study was needed to obtain more definite conclusions about the potency of the suspensions over time. Though no limits were set for the potency of the suspension to be incorporated into DPTPolio, the NVP-96 suspensions expired 2 years after production. In practice, rarely suspensions older than 12 months were used in DPTPolio. Consequently the MPT was done in duplicate on the NVP-96 and NVP-THIJS suspensions every 6 months for 2 years. The data for the NVP-96 and NVP-THIJS suspensions for strain 134 and 509 are shown in Figure 4. For most vaccines, pertussis vaccines included, the potency decreases over time. The trend line is based on the potency values, as well as the weighed geometric mean of the confidence intervals of these potency values. The slope of the line plotted through the data is directly proportional to the stability of the suspensions tested. From Figure 4A and 4B the slopes of the plotted lines are with 95% certainty identical for all suspensions, indicating that the stability is similar for both the NVP-96 and NVP-THIJS suspensions, regardless of strain. However, due to the higher initial potency of the THIJS-suspensions, the potency after 2 years storage also remained higher. The difference in potency between the strain 134 suspensions was statistically significant, while for the potency of the strain 509 suspensions this was not the case.

4. Discussion

In the previous paragraphs the production process of a cellular vaccine was examined and rationally optimised to increase consistency and potency. The most important factor in the standardisation and optimisation was the production culture, since this is where the antigen content of the bacteria is determined. The consistency of production markedly increased for the NVP-96 and NVP-THIJS process as compared to the NVP process based on the reduction in variation of antigen content as measured with ELISA and the reduction in variation in the MPT. Furthermore, the use of a chemically defined medium greatly enhanced the consistency of the production culture, as witnessed by the consistent reproducible course in time of the culture parameters.



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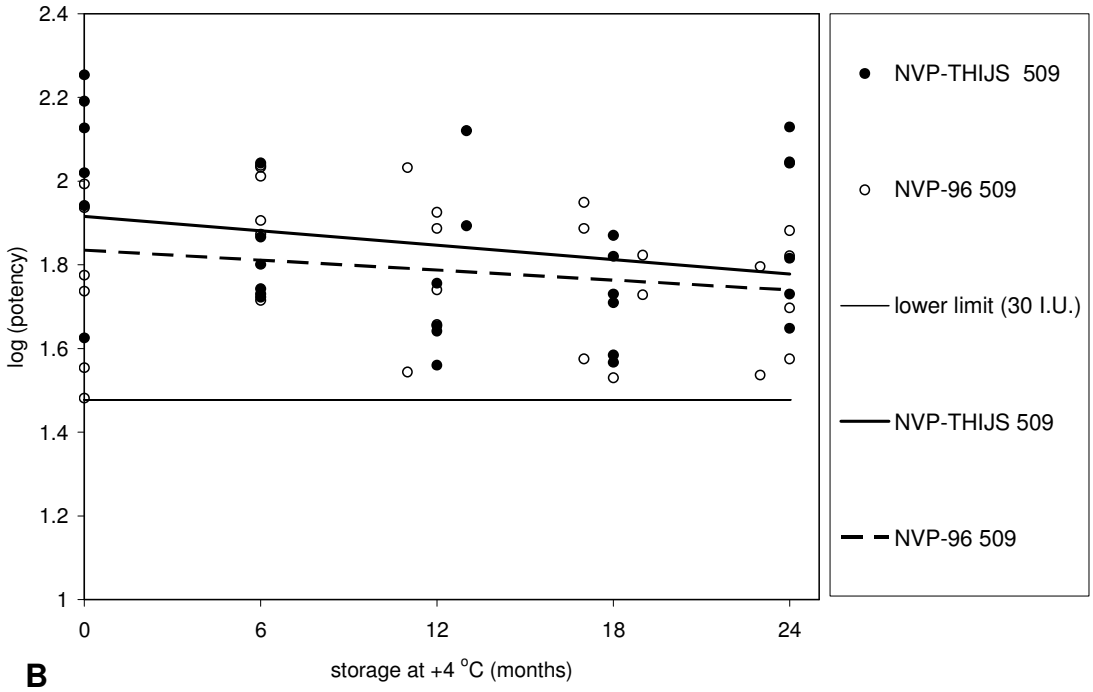

B

Figure 4. MPT values of NVP-96 and NVP-THIJS suspensions for strain 134 (A) and strain 509 (B) over a 2-year period. The plotted line was calculated taking the confidence limits of the potency values into account. Note that the scale is logarithmic.

Due to the inaccuracy of the MPT, no statistical significant differences in the potency of the NVP, NVP-96 and NVP-THIJS suspensions could be detected by the MPT, even though the average potency of the NVP-96 and NVP-THIJS products is 2 and 4 fold higher, respectively, than the NVP product. Also, the antigen content as measured with an ELISA [17], which is likely to be linked to potency, could not detect statistically significant differences in antigen content between the 3 types of products. However, this may be due to the indirect nature of the ELISA. On the other hand, the MWG serological test was able to show statistically significant differences between the NVP-96 and the NVP-THIJS suspensions. The immunogenicity of NVP-THIJS was shown to be 2 to 3-fold higher for strain 509 and 134, respectively, which agrees with the higher values obtained for the potency in the MPT.

None of the in process controls used in this study clearly identified why the immunogenicity in the MWGT and the potency in the MPT of the THIJS suspensions were higher than those of the NVP-96 suspensions. Given the fact that the strains used for both processes are identical, the difference can only lie in the amount of antigens expressed per unit biomass, the degree of degradation of the antigens present or a combination of both. The short culture time in the THIJS medium as well as harvesting the cells before reaching the stationary phase, results in limited antigen degradation or shedding of antigens into the culture medium. Additionally, the short time between harvest and inactivation will further limit the degradation of antigens. Without conclusive data to substantiate these assumptions, it is quite possible that the THIJS suspensions not only contain more antigen per human dose, but the antigens present are possibly more intact as well.

Assuming the NVP-THIJS product is 2 to 3 times as immunogenic in humans as the NVP-96 product, it could theoretically be applied at a half the current vaccine dose, and still result in a potency comparable to that of the NVP-96 product. Whereas a 10% reduction in LPS content may be hard to detect in a field trial, a 50% reduction should result in less adverse reactions, even in a limited clinical trial. Since these studies are difficult to carry out, we are currently evaluating a rabbit model to predict fever in infants, the most common and most quantifiable adverse event.

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CHAPTER 5

Fed-batch cultivation of *Bordetella pertussis*: metabolism and Pertussis Toxin production

Summary

The production of acellular pertussis in comparison with whole cell pertussis vaccines demands 5 to 25 times the amount of *B. pertussis'* virulence factors such as pertussis toxin (PT), to produce the same number of vaccine doses. An increase in the volumetric productivity by employing fed-batch rather than the currently used batch cultivations of *B. pertussis* could reduce the cost price of acellular pertussis vaccines. This study defined the conditions that enable fed batch cultivations at high specific PT production. A solution containing lactate and glutamate was fed to the cultures at various rates. The feed rate and whether or not the fed substrates were completely consumed, significantly influenced cellular metabolism. If lactate was detectable in the culture broth while glutamate was not, poly-hydroxy-butyrate (PHB) was formed. Any PHB present was metabolized when glutamate became detectable again in the culture liquid. At higher lactate and glutamate concentrations, free fatty acids were produced. Though toxic, free fatty acids were not the reason cultures stopped growing. By choosing appropriate conditions, a cell density of 6.5 $g.L^{-1}$ dry weight was reached, i.e. a 7-fold increase compared to batch culture. The metabolic mechanisms behind the formation of PHB and fatty acids are discussed, as well as how to further increase the cell density. The PT production stopped at 12 $mg.L^{-1}$, well before growth stopped, indicating that regulatory mechanisms of PT production may be involved.

key words: acellular, fed batch, metabolism, pertussis toxin

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1. Introduction

Whooping cough is caused by the Gram-negative bacterium *Bordetella pertussis,* a highly contagious disease that mainly infects infants and young children. The most commonly used vaccine against whooping cough consists of inactivated whole cells of *B. pertussis*. A consequence of the use of inactivated whole cells is that adverse reactions occur after vaccination due to the lipo-poly-saccharide (LPS) present in the bacterial membrane. This led to the development of acellular vaccines, consisting of 1 to 5 protein virulence factors that cause less adverse reactions such as fever and pain at the injection site. A comparison of the composition of various acellular vaccines [1] and the amount of the various antigens in a cellular vaccine [2] shows that the Pertussis Toxin (PT) content in an acellular vaccine is 5 to 25 fold higher than in a cellular vaccine. The other virulence factors such as filamentous heamagglutinin (FHA), pertactin (PRN) and fimbriae (FIM) occur in 2 or 3-fold higher concentrations in the acellular vaccine than in the cellular vaccine. For this reason, PT is the bottleneck in the production of an acellular vaccine. If the current batch cultivation technology used for cellular vaccine production is also used to produce acellular vaccine, the cultivation capacity has to increase accordingly.

While the cultivation of *B. pertussis* is only a fraction of the total manufacturing costs of a cellular vaccine, this changes if the number of cultivations to produce the same number of doses of an acellular vaccine increases 5 to 25-fold. One way to meet the requirement for increased production and to limit the increase in price of a pertussis vaccine when changing from cellular to acellular vaccine production, is to employ fed-batch rather than batch cultivation. Fed-batch cultures generally lead to higher cell densities and usually to higher product concentrations if the fed batch process is successful. In order to develop a fed batch cultivation, the essential parameters to optimize are the feed composition and the feed rate. The feed composition should balance the catabolic and anabolic requirements of the organism in order to prevent accumulation of specific added substrates or metabolites formed. The demand for cellular building blocks such as amino acids, saccharides, fatty acids and nucleotides, as well as the demand for energy and reducing power to produce these building blocks may change with the growth rate. This could in turn lead to changes in the metabolic flux distributions and consequently, the feed composition would have to change along with the growth rate to remain balanced. Since the growth rate is set by the value of the feed rate, the speed with which the feed is fed to the bioreactor is of crucial importance. If the feed-rate is low, the growth rate is also low, which means that the percentage of substrates used for maintenance may be relatively large. Not only will the yield of biomass per C-mole be low in this case, also the N:C ratio of the medium substrates may need adjustment since maintenance processes primarily serve to

generate ATP, without consumption of nitrogen. Although a high feed rate on the other hand allows bacteria to grow faster, too high feed rates will result in high substrate levels, which can lead to excretion of metabolites or cause the growth rate to decline [3]. For these reasons finding the correct feed composition in combination with the optimal feed rate regime is of critical importance for the result of the fed-batch process.

The aim of the work described in this paper was to study the influence of the feeding regime in fed-batch cultivation on the metabolism and growth characteristics of *B. pertussis*. For this biomass, lactate, glutamate, PHB, PT, as well as the total and free fatty acids content were measured. The feed composition was derived from the batch medium. In batch cultures *B. pertussis* grows on THIJS medium with a glutamate to lactate molar ratio of 1:1.6 in a balanced way, i.e. no waste metabolism occurs until these substrates are completely consumed [3]. Therefore, the feed composition used here contains the same ratio of the substrates and the same amount growth factors as the batch medium but in a concentrated form.

1.1 *B. pertussis'* metabolism

In order to understand the effect of the feeding regime on *B. pertussis*' growth characteristics and metabolism, a thorough understanding of *B. pertussis'* basic metabolism is required. As most pathogenic organisms, *B. pertussis* has limited metabolic capabilities. *B. pertussis'* basic metabolism was studied by a number of authors [3,4,5]. In Figure 1, the main metabolic pathways of *B. pertussis* are shown, including the main metabolites and the cofactors involved in the various reactions. As far as reactions, intermediates of cofactors could not be directly derived from
B. pertussis literature or the genome database, we used standard biochemical handbook information to complete the pathway.

B. pertussis does not possess a functional glycolysis (Fig. 1, reaction 1 and pathway 2) due to a deletion of 2 genes in the glycolytic pathway [6]. Phospho-enol-pyruvate (PEP), normally an intermediate of the glycolysis, can be formed from glutamate in *B. pertussis* (Fig.1, reaction 12, pathway 11b, followed by reaction 7a). PEP can be converted to glucose-6-phosphate through the gluconeogenesis pathway (Fig. 1, pathway 2). The genes for this pathway are intact [6], and the activity of the pathway is confirmed by the observation that *B. pertussis* is able to form saccharides for lipo-poly-saccharide (LPS). The pentose phosphate pathway (PPP) must also be intact, since *B. pertussis* can form nucleotides from glutamate or lactate (Fig. 1, pathway, 13d) and can oxidize lactate and glutamate mixtures to carbon dioxide (Fig. 1, pathway 3b) [3].

Lactate, one of the main substrates in the THIJS medium, can be converted into pyruvate (Fig.1, reaction 6). Pyruvate serves as a carbon backbone for the synthesis of several amino acids (Fig. 1, pathway 13a), but can also enter gluconeogenesis

(Fig. 1, reaction 7b, pathway 2). While pyruvate itself is toxic in millimolar amounts, lactate is not; concentrations of lactate up to 60 mM do not hamper growth. Similar concentrations of glutamate, however, lower the growth rate [3]. Finally, pyruvate can be oxidized to an acetyl-moiety covalently linked to Co-enzyme A (CoA) (Fig. 1, reaction 8).

In normal aerobic metabolism, the acetyl-moiety of acetylCoA can be oxidized in the citric acid cycle (Fig.1, pathway 11). Although all genes of the citric acid cycle are present in *B. pertussis* [6], the citric acid cycle is not fully functional [3] (Fig.1, pathway 11a). This discrepancy may be explained by the fact that although the genes of the citric acid cycle are present, this does not necessarily mean the corresponding enzymes are expressed. The citric acid 'cycle' is functional from α-ketoglutarate onward (Fig. 1, pathway 11b).

AcetylCoA can serve as building block for fatty acid synthesis, eventually to be incorporated into lipids (Fig. 1, pathway 9a through 9b) in normal metabolism. However, the formation of fatty acids (Fig. 1, pathway 9a) that are not incorporated into lipids, has also been reported for *B. pertussis* [7]. These free fatty acids (FFA) can be toxic to the organism in the sense that growth is inhibited when micro molar concentrations of palmitate are added to the medium (Frohlich, 1996). It is not clear why or under what conditions fatty acids formed are not esterified to form lipids. Even though these FFA's are not esterified to lipids, they are incorporated into the membrane, which has been reported to contain up to 10% of FFA's [7].

The other fate of acetylCoA in *B. pertussis* is incorporation into poly-hydroxy-butyrate (PHB) (Fig. 1, pathway 10a to 10b) releasing 2 CoA molecules per monomer hydroxy-butyrate added. PHB acts as a storage polymer, since it can be metabolized again later (Fig.1, pathway 10b to 10a) [3]. Given the fact that PHB can occupy a significant amount of cell volume [3], it is important to determine and control the currently unknown conditions that determine PHB formation and consumption. While PHB forms globules in the cell, these do not appear to be toxic to the organism.

Energy generation during normal and waste metabolism. The combination of the gluconeogenesis pathway(Fig.1, pathway 3) and the PPP can be used by *B. pertussis*, to generate either 1 ribose-5P and 2 NADPH or 12 NADPH and 6 CO_2 (Fig. 1, pathway 3a or 3b respectively). While NADPH is used only for biosynthetic reactions and cannot be used to generate ATP, many organisms including *B.*

pertussis [6], possess transhydrogenases that can convert NADPH into NADH, that can in turn be used to generate approximately 1 ATP per NADH. Therefore *B. pertussis* can oxidize lactate and glutamate to carbon dioxide, leading to ATP yields of 1.2 to 1.4 ATP/C-mole (Table 1). For comparison; the ATP yield for both glutamate and lactate oxidized in the citric acid cycle is 2.0 ATP/C-mole carbon.

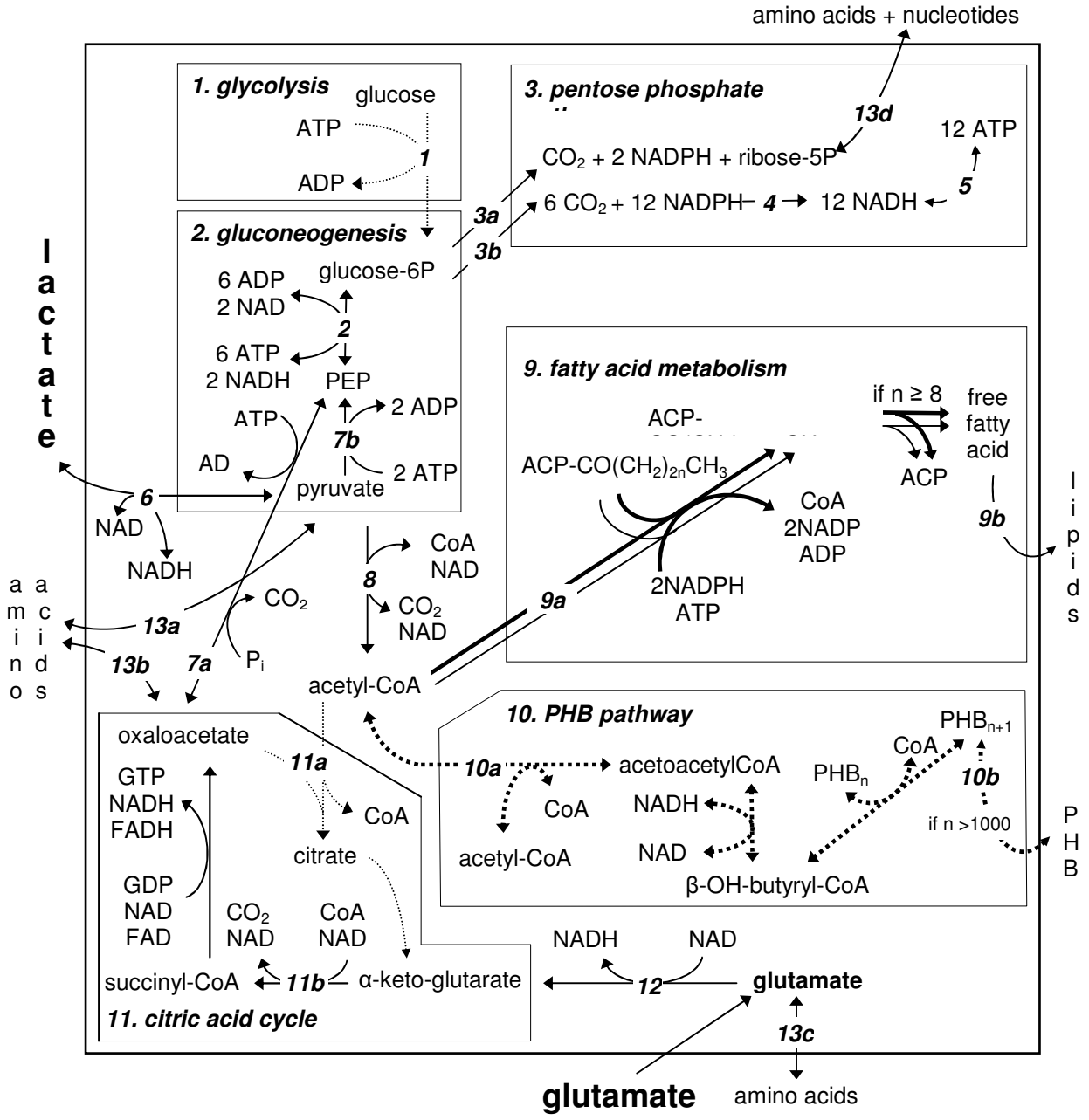

Figure 1. Overview of the functional (solid lines) and dysfunctional (dotted lines) metabolic pathways or reactions present in *B. pertussis*, only showing the main metabolites and the co-factors involved. The different line-types indicate *B. pertussis*' metabolism during: balanced biomass formation (thin solid lines), PHB formation (bold dotted lines) or excess fatty acid formation (bold solid lines). The main substrates in the THIJS medium (Thalen, 2005) are indicated in large bold print. The PPP is shown operating at the 2 extremes: maximum NADPH generation (pathway 3b) in conjunction with gluconeogenesis and maximum ribose-5P formation (pathway 3a).

If lactate is converted into PHB, 1.5 NADH is formed (Table 1), which can be rapidly regenerated in order to make ATP (0.5 ATP/C-mole lactate). Hydrolyzing PHB probably yields 1 NADH per hydrolyzed PHB-monomer, or 0.16 ATP/C-mole lactate incorporated into PHB. The theoretical ATP yield for PHB generation and breakdown from lactate is therefore 0.66 ATP/C-mole. Although *B. pertussis* cannot grow on lactate alone [3], it can still generate ATP. Therefore in the presence of excess lactate, the organism can generate NADH and store carbon and energy in the form of PHB. PHB formation from glutamate yields 7 NADH, 4 ATP and 2 FADH (Table 1). While NADH can be readily regenerated to yield ATP, it is unclear if FADH can be used to generate ATP in *B. pertussis*. The theoretical ATP yield for PHB generation and breakdown from glutamate is therefore estimated to be 1.1 ATP/C-mole (Table 1).

Table 1. Metabolism of lactate and glutamate in complete oxidation, PHB and fatty acid formation. The overall stoichiometry of the various metabolic processes is given, as is the ATP yield, assuming the presence of a transhydrogenase and a conversion of 1 NAD(P)H to 1 ATP, and that FADH does not give rise to ATP formation.

process	pathways (Fig.1)	overall stoichiometry		ATP yield (ATP/C-mole)
oxidation	6, 2, 3a	2lactate + 6ATP+ 12NADP	$\Rightarrow$ 6ADP + 12NADPH + $6CO_2$	≈ 1.0
	12, 11b, 7a, 2, 3a	2glutamate + 4NAD + 2NADP + 2FAD	$\Rightarrow$ 4NADH + 12NADPH + $10CO_2$ + $2FADH_2$	≈ 1.6
PHB formation	6, 8, 10	2lactate + 3NAD + PHB_n	$\Rightarrow$ 3NADH + PHB_{n+1}	≈ 0.5
		2glutamate + PHB_n + 7NAD + 2FAD + 4ADP	$\Rightarrow$ PHB_{n+1} + 7NADH + 2FADH + 4ATP + $2NH_3$ + $6CO_2$	≈ 1.1
fatty acid formation	6, 8, 9	lactate + 2NAD + 2NADPH + ATP + ACP- $CO-(CH_2)_{2n}-CH_3$	$\Rightarrow$ ACP-$CO-(CH_2)_{2n+1}-CH_3$ + 2NADH + 2NADP + ADP + CO_2	≈ - 0.3
	12, 11b, 7a, 8	glutamate + FAD + 4 NAD + ADP + 2 NADPH + ACP-$CO-(CH_2)_{2n}-CH_3$	$\Rightarrow$ ACP-$CO-(CH_2)_{2n+1}-CH_3$ + 4NADH + 2NADP + ATP + FADH + NH_3 + $3CO_2$	≈ 0.6

The stoichiometry of the pathways for fatty acid formation from lactate and glutamate is also shown in Table 1. The formation of fatty acids from lactate requires a net input of energy, and is therefore not suitable as a pathway to generate energy. From an energetic perspective, excess of lactate is therefore unlikely to lead to free fatty acid formation. Generation of fatty acids from glutamate however generates 2 NADH and 1 ATP, and could in theory be used as a pathway to generate energy (Table 1).

2. Material and Methods

Strain. *B. pertussis* strain 509 (NVI collection, Bilthoven, the Netherlands), one of the two strains included in the DPT-Polio vaccine in the Netherlands, was used in all experiments.
Batch and feed media. The batch medium used was the same in all cultivations [8]. In some experiments 1 g/L cyclo-dextrin was added as indicated in the text. The composition of the feed medium was the same in all experiments. Two concentrated stock solutions of 0.510 M glutamate and 0.855 M lactic acid were used as separate feeds, since glutamate precipitates in the presence of lactic acid. The lactate solution also contained 250 ml/L Stainer-Scholte supplement [9]. Both stock solutions were added simultaneously at the same volumetric rate.
Bioreactor conditions and operation. The cells were grown in a 5 L round-bottomed glass bioreactor containing 3 L medium. An eight-bladed marine impeller was used to agitate the medium. Temperature, pH, dissolved oxygen, and stirrer speed were controlled at 34ºC, 7.2, 20%, and 500 rpm, respectively. Oxygen was transported through the headspace only and controlled by changing the oxygen fraction in the gas flow. The total gas flow was maintained at 1 L/minute. The pH was maintained using 1 M HCl. Both the HCl bottle and the bottles containing the feed solutions were placed on scales to monitor the amount added. A polarographic electrode (Ingold, Urdorf, Switzerland) measured the dissolved oxygen in the medium and a pH electrode (Ingold) the pH. The temperature was measured with a Pt100 temperature sensor (ADI, Schiedam, the Netherlands).The feeds were added to the bioreactor by two pumps (101U/R 32 rpm, Watson Marlow Ltd., Cornwall, UK) at the same flow rate, controlled by the bioreactor control system. The Hard- and Software set-up was as described before by [10]. Briefly: all sensors were connected to the bioreactor control system ADI1040 (Applikon, Schiedam, The Netherlands), which in turn was connected to a UNIX machine with BCSV (Compex, Belgium). All standard control-loops (dissolved oxygen, pH, temperature, etc.) were performed in the BCSV-software and logged to a disc on the UNIX machine. Exponential growth rates were programmed into the BCSV-software, using the growth rate and the initial percentage pump flow as parameters. The exponential feeds started at 20% of the maximum, flow rate of 160 mmol total C/hour.
Biomass and metabolite determinations. Biomass concentrations were determined by measuring the optical density at 590 nm, using a Vitalab 10 (Vital Scientific, Dieren, The Netherlands), and by dry weight as described before [3]. Optical density and dry weight content were always measured independently. Glutamate and L-lactate were determined with a YSI 2750 Select analyzer (Yellow Springs Instruments, Yellow Springs, USA).
Amino acid composition of B. pertussis. Culture samples of 2 to 5 ml, depending on the cell density, were centrifuged at 8000 g for 10 minutes. The cell pellets were resuspended in 2 ml saline to which 2 ml of 12 M HCl was added, in glass

tubes with Teflon coated screw caps. The tubes were kept at 110 ^{o}C for 20 hours, after which 4 ml of 6 M NaOH was added. Amino acids were determined using an HPLC method as described before [11]. The amino acid composition of the following *B. pertussis* genes were used to compare to the amino acid composition of hydrolyzed cells: Mn sod, ompQ, B. p porin, EPSP synthetase, fim C, fim D adhesin, fim3 precursor, TcfA, 93kD PRN precursor, dermonecrotic toxin, adenylate cyclase, PT, FHA-D, FHA-A, FHA-E, and FHA-B.
Isolation of lipids, fatty acids, PHB and NMR analysis. As described before [3].
Analysis of free and esterified fatty acids. As described before by [12].
PT and proteolytic-activity determination . The method of [13] was used, using the PT standard JNIH 90/518.

3. Results and Discussion

3.1 Constant feed rates

In order to investigate the magnitude of the effects of the feed rate on the final cell density, two identical cultures were given the same feed, but set at different rates, when the optical density reached 1.5 or higher. The low feed rate was 10 mmol $C.L^{-1}.h^{-1}$, which corresponds with the consumption of substrates at OD 1.5. The high feed rate was 45 mmol C. $L^{-1}.h^{-1}$, a feed rate that cannot be totally consumed by the organism at that point in the cultivation, which should result in high substrate concentrations during the feed phase. The results of these cultures are summarized in Table 2. The bioreactor receiving a low feed rate reached a final optical density of 8.2 after 72 hours, or a four-fold increase in cell density compared to 2.0 OD normally reached at the end of batch culture. The optical density of the culture receiving the high feed rate however only increased to 3.3 OD. The substrate levels in the culture supernatant of this culture steadily increased, while no substrates were detectable in the supernatant of the culture receiving the low feed rate (Table 2). The high glutamate level in the culture receiving the high feed rate may have contributed to the low growth rate [3]. Although the culture receiving the low feed rate reached a 2.5 times higher cell density than the culture receiving the high feed-rate, the total maximum PT concentration was only 30% higher. Therefore the PT produced is not directly related to the amount of biomass present.

Whereas both cultures had a similar biomass yield at the end of the batch phase, the culture receiving the high feed rate showed a significant decrease in biomass yield at the end of the feed phase (Table 2). However, no waste products were excreted into the medium (no data shown). Also, the total amount of PHB formed in both cultures is low in relation to the total amount of biomass, although it was 15-fold higher in the culture receiving the high feed rate. Apparently PHB itself was not formed as a significant waste product under these circumstances.

Table 2. Summary of constant feed rate cultures.

	low feed rate		**high feed rate**	
parameter at the end of:	**batch**	**fed-batch**	**batch**	**fed-batch**
feed rate (mmol C.l^{-1}.h^{-1})	-	10	-	45
cell density (optical density)	1.5	8.2	1.8	3.3
total culture time (hours)	19	72	22	42
overall yield (g. C-mole^{-1})	9.8	7.0	9.1	4.2
[lactate] (mM)	4.6	0	2.2	69
[glutamate] (mM)	4.5	0	2.8	72
maximum PT (mg/L)	5.3		4.0	
maximum free fatty acids (% of total fatty acids)	8.7		13.8	
maximum PHB (% of dry weight)	0.06		1.01	

The results of the free fatty acids analysis showed these primarily consisted of saturated (C16:0) palmitate, unsaturated (C16:1) palmitoleate and stearate (C18:0) (no data shown), which is in agreement with [14]. In order to quantify the potential toxicity of the free fatty acids, they are expressed in Table 2 as a percentage of the total fatty acids, free and esterified. For both cultures the maximum percentage of free fatty acids did not coincide with the end of the culture. Apparently, the 9 to 14% free fatty acids attained here were not the reason the cultures stopped growing.

The differences in biomass and PT yield warrant further investigation into the effect of the feed rate on the metabolism of the organism. Since the feeds used were constant, the growth rate was declining as the cell density increased, which could also influence the composition of the cells. From an experimental point of view, it is better to use exponential feeds to keep the growth rate constant. Also, [8,15] showed that PT is not only formed, but also degraded during a *B. pertussis* cultivation. Shortening the cultivation period should therefore also reduce the amount of degraded PT, i.e. the exponential feed rate should preferably be high.

3.2 Exponential feed rates

Exponential feed rates enabling a μ = 0.11, 0.09 and 0.07 h^{-1} were applied to the *B. pertussis* cultures shown in Figure 2, which is substantially below the maximum growth rate of 0.16 h^{-1} on this medium [10]. The culture receiving a feed rate enabling a μ of 0.07 h^{-1} reached a final Optical Density of 13.6 (6.5 g/L dry weight, data not shown), an almost 7-fold increase of the batch culture. The substrate levels of this culture were below the detection limit for most of the culture. After 44 hours, however, the lactate level rose to 0.43 mM (Fig. 2, right dashed arrow), while the glutamate level rose to 0.16 mM after 53 hours of cultivation (Fig. 2B, right dotted arrow). The final cell density reached for the exponentially fed cultures enabling a μ of 0.11 and 0.09 h^{-1} was substantially lower at 7.3 and 5.9 O.D. units, respectively (Figure 2A). Figure 2B shows that the culture that received a feed-rate of 0.11 h^{-1} contained substrates in the millimolar range throughout the culture. However, the culture did not grow at the maximal growth rate of 0.16 h^{-1} (no data shown). The culture that received a feed rate of 0.09 h^{-1}, initially consumed all supplied substrates. However, lactate could be detected after 25 hours (Fig. 2, left dashed arrow) and glutamate could be detected after 33 hours (Fig. 2B, left dotted arrow) hours of cultivation.

Judging from the increase in substrate levels in Figure 2, the feed rates based on growth rates of 0.11 or 0.09 h^{-1} are too high for *B. pertussis* to maintain in a fed-batch cultivation. At a growth rate of 0.07, the organism could consume the supplied substrates longer, but from 50 hours onwards the organisms in this culture could also no longer consume all substrates supplied. An explanation for the fact that the organism apparently cannot continue to grow at high growth rates may be that with the addition of the feed, Na^+ is added in the form of Na-glutamate. Thalen *et al* [8] showed that the maximum growth rate of *B. pertussis* batch cultures depended strongly on the [Na^+]. It is quite likely that the increasing Na^+ concentration lowers the maximum growth rate of the organisms, since for example approximately 95 mM Na^+ was added in the feed phase to the cultures that grew with 0.07 h^{-1}. At a certain point in the feed phase, the [Na^+] in the bioreactor reaches the point where the feed rate exceeds the maximum growth rate at that given [Na^+]. Thus the organism could at that point no longer consume all supplied substrates, which leads to high Na-glutamate concentrations, which lowers the growth rate even further.

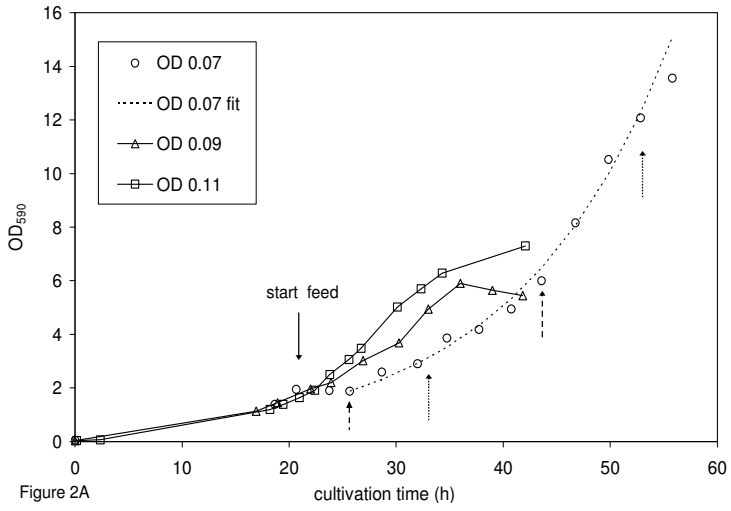

Figure 2A

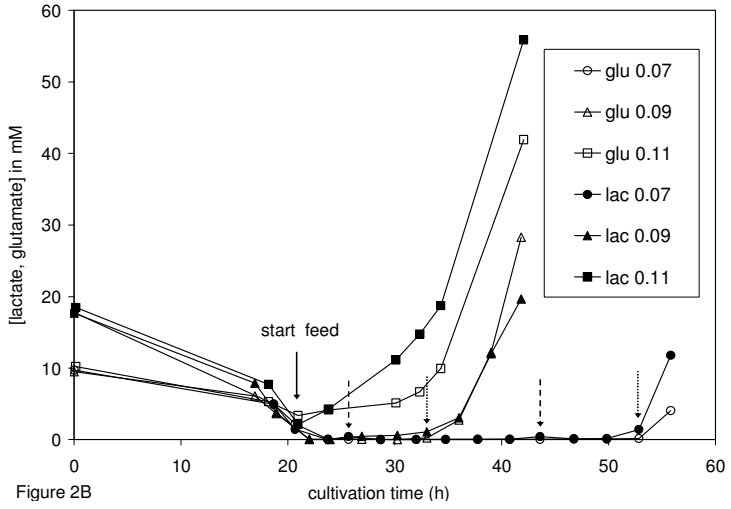

Figure 2B

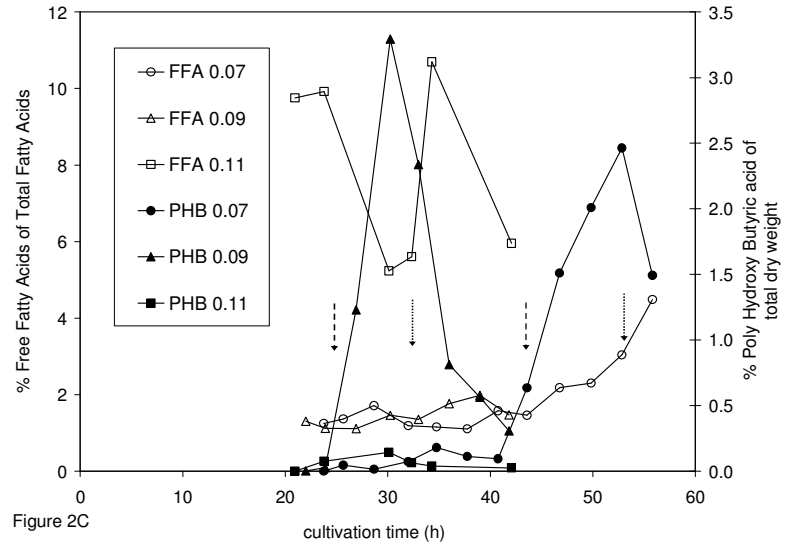

Figure 2C

Figure 2. A: Optical density and substrate concentrations of 3 cultures that were given a feed at a rate enabling a growth rate of 0.07, 0.09 and 0.11 h^{-1}, respectively. B: The levels of the substrates lactate and glutamate of the cultures in figure 2A. The solid arrow indicates the start of the feed addition, the dashed arrows indicate incomplete consumption of added lactate, while the dotted arrows indicate the incomplete consumption of both lactate and added glutamate. C: PHB content in percentage of the total dry weight, and percentage free fatty acid of the total fatty acid content of the cells.

3.3. Flux through acetylCoA: PHB formation

The high substrate concentrations of both lactate and glutamate for the culture receiving the feed rate of 0.11 h^{-1} (Figure 2B), did not lead to intracellular PHB accumulation. The culture receiving a feed rate 0.09 h^{-1} however, showed a peak accumulation of PHB up to 3% of the total biomass, indicating a high flux through the PHB pathway (Fig. 1B, pathway 10a). The rise in PHB coincided with the appearance of lactate in the culture supernatant (Fig. 2, left dashed arrow), while glutamate was not detectable. The decrease of the cellular PHB content for this culture (Fig. 1B, pathway 10b) coincided with the appearance of glutamate in the culture supernatant, in the presence of lactate (Fig. 2, left dotted arrow). The culture receiving a feed rate of 0.07 h^{-1} showed a similar pattern: PHB formation coincided with the appearance of lactate in the medium, while breakdown of PHB was seen after glutamate became detectable in the medium.

To verify if this pattern was also present for the other cultures discussed in this study, for each culture the production and breakdown of PHB was evaluated for three separate periods during the culture where, respectively, lactate and glutamate levels were low ([glutamate] and [lactate] ≈ 0), excess lactate and glutamate was present ([glutamate] and [lactate] > 0), and lactate was present, but the glutamate level was low (([glutamate] ≈ 0, [lactate] > 0). Figure 3 shows that virtually no PHB was formed when both glutamate and lactate levels were low (Fig 3, circles). When glutamate and lactate were both present, any PHB present was degraded (Fig. 3, squares). When lactate was present in the absence of glutamate, PHB was formed (Fig. 3, triangles). Therefore, it can be concluded that the presence of lactate in the absence of glutamate causes PHB formation, while any PHB present is degraded when glutamate is present (Fig. 1, pathway 10). In the absence of glutamate, *B. pertussis* cannot grow. Table 1 shows that it is useful to form PHB since it not only generates a useful storage polymer, but also 0.5 ATP C-$mole^{-1}$ in the process.

A culture that unintentionally grew oxygen limited also gave rise to PHB formation (Fig. 3 X-symbols). The data of this culture was not used further in this study. The oxidation of glutamate to for example pyruvate yields 4 NADH and 1 FADH (Fig. 1, pathways 12, 11b and 7a) that cannot be regenerated easily under oxygen limited conditions. Therefore, the oxidation of glutamate will be limited as well, which can be compared to the situation when glutamate levels are low in the presence of excess lactate. Under these conditions the organism can probably still metabolize lactate into PHB since this pathway (Fig. 1, pathways 6, 8 and 10a) only generates 1.5 NADH per lactate used. Therefore the acetylCoA units for the formation of PHB under oxygen-limited conditions were probably derived from lactate rather than from glutamate.

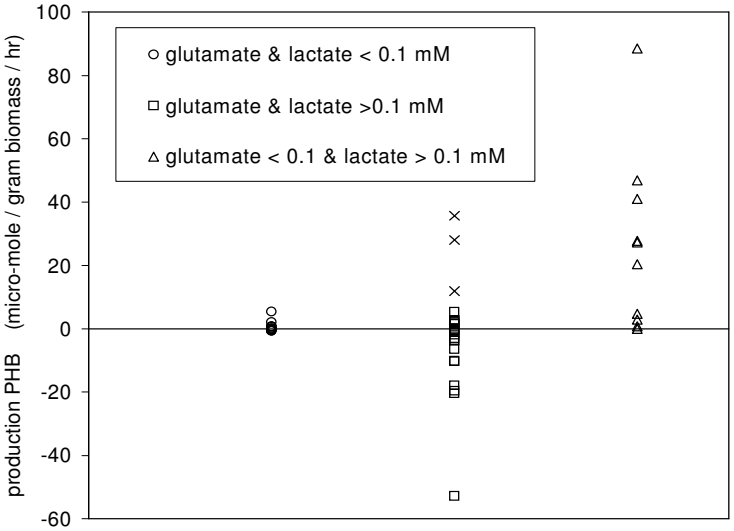

Figure 3. Influence of substrate concentrations on production and breakdown of PHB. Production or degradation was determined relative to the previous point in time of the cultivation. For the X-symbols see text.

3.4. Flux through acetylCoA: free fatty acid formation

The culture receiving a feed-rate of 0.07 h^{-1} contained 2% or less free fatty acid , until both glutamate and lactate became detectable. From this point, i.e. from 50 hours onward, the free fatty acid content increased from 2.3 to 4.5%. The free fatty acid percentage of the culture receiving a feed rate of 0.11 h^{-1} varied between 5 and 11%, while substrate levels were continuously in the millimolar range. The culture receiving a constant high feed rate had a high free fatty acid content and high substrate levels. This suggests a correlation between high substrate levels and formation of free fatty acids. However, the percentage of free fatty acids in the membrane for the culture receiving a feed rate of 0.09 h^{-1} averaged less than 2% of the total fatty acid content during the whole culture period (Figure 2B), also when the substrate levels started rising after 35 hours of cultivation. Notably this culture reached a lower biomass density than the culture grown at 0,11 h^{-1}, which had a higher free fatty acid content. This suggests that free fatty acids were not inhibiting growth. To check this, a culture was run at the same conditions as in Figure 2, at a feed rate of 0.07 h^{-1}, in the presence of 1 g/L cyclo-dextrin. Cyclo-dextrin, a commonly used heptamer of glucose [16], can bind free fatty acids. Thus if free fatty acids were the cause of cessation of growth of the culture receiving a feed rate of 0.07 h^{-1} in Figure 2, then a cultivation in the presence of cyclo-dextrin should reach a higher final cell density. The final optical density of 13.0 (5.8 g/L dry weight) and the percentage of free fatty acids were comparable to the culture without cyclo-dextrin (no data shown). The substrate

levels were below detectable levels until the end of the culture, when lactate and glutamate levels rose simultaneously. If 1 g/L cyclo-dextrin is enough to bind all free fatty acids, this confirms that free fatty acids formation was not the reason the cultures stopped growing, which is consistent with the results of [14]. Thus, there is another reason the cultures stopped growing, which seems to cause high substrate levels and high free fatty acid levels, rather than free fatty acid levels inhibiting the cultures from growing further.

3.5 Feed rates and amino acid composition of *B. pertussis* cells

In order to investigate whether the different culture conditions influence the relative amino acid composition of the cells, cell culture samples from the cultures above were divided in three categories. These correspond to conditions where cells can grow substrate limited ([glutamate] and [lactate] ≈ 0), in the presence of excess lactate and glutamate ([glutamate] and [lactate] > 0), and glutamate limited (([glutamate] ≈ 0, [lactate] > 0), respectively. The cell samples were chemically hydrolyzed, and the resulting amino acids were measured. As shown in Figure 4, there was no clear difference between the three categories. While the relative amounts of tryptophan and cysteine could not be measured, it seems unlikely that the relative amounts of these amino acids would vary from sample to sample while all others remained the same. As a theoretical composition of *B. pertussis*, the amino acids composition of 15 *B. pertussis*' proteins present in gene banks were used, representing a significant amount of the total protein produced by *B. pertussis*. This theoretical composition of the proteins of *B. pertussis* and the measured composition *B. pertussis* cells were quite similar which is to be expected if the total number of amino acids becomes large enough.

From the data in Figure 4 it is clear that the relative amino acid composition of *B. pertussis* cells does not change significantly due to culture conditions. Thus, the relative metabolic fluxes through the anabolic pathways of amino acid synthesis (Figure 1, pathways 13a, b and c) are not significantly influenced by growth rate or culture conditions. Therefore the flux through these pathways does not seem to be an important parameter to monitor during the development of a fed-batch cultivation. In addition, with respect to protein formation the feed composition can be kept constant for growth rates between 0.07 and 0.11 h^{-1}.

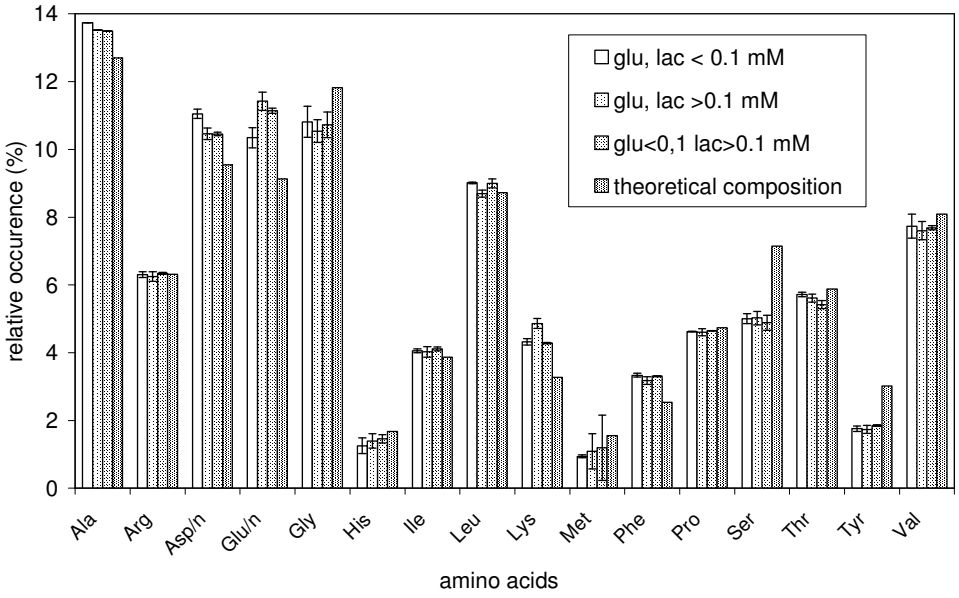

Figure 4. Amino acid composition of *B. pertussis* cells grown under various culture conditions. The theoretical relative composition of *B. pertussis* cells was derived from the amino acid composition of 15 *B. pertussis* proteins.

3.6 Feed rates and Pertussis Toxin production

In Figure 5 the total PT content of the culture liquid was plotted against the optical density, for the exponentially fed cultures discussed above. The PT content at the end of the batch phase is approximately 2.0 ±0.18 mg/L, or 2.2 ±0.29 mg/g biomass (n=5), for all cultures except for the culture that contained cyclodextrin (Fig. 5, triangle), which had a higher PT content of about 5.0 mg/l. During the first part of the fed-batch phase, the PT production was growth associated (Fig. 5 closed symbols). However, at a certain moment PT production stopped, while growth still continued (Fig.5, open symbols). The cultures that received a feed-rate of 0.09 and 0.11 h^{-1} (Fig. 5, squares and diamonds respectively) almost increased 50% in density after PT production had stopped. Both cultures growing at a feed-rate of 0.07 (Fig. 5, triangles and circles) increased 30% after PT production had stopped. These findings on the defined medium used here are in contrast with [17], who found that in complex medium PT production was always growth associated.

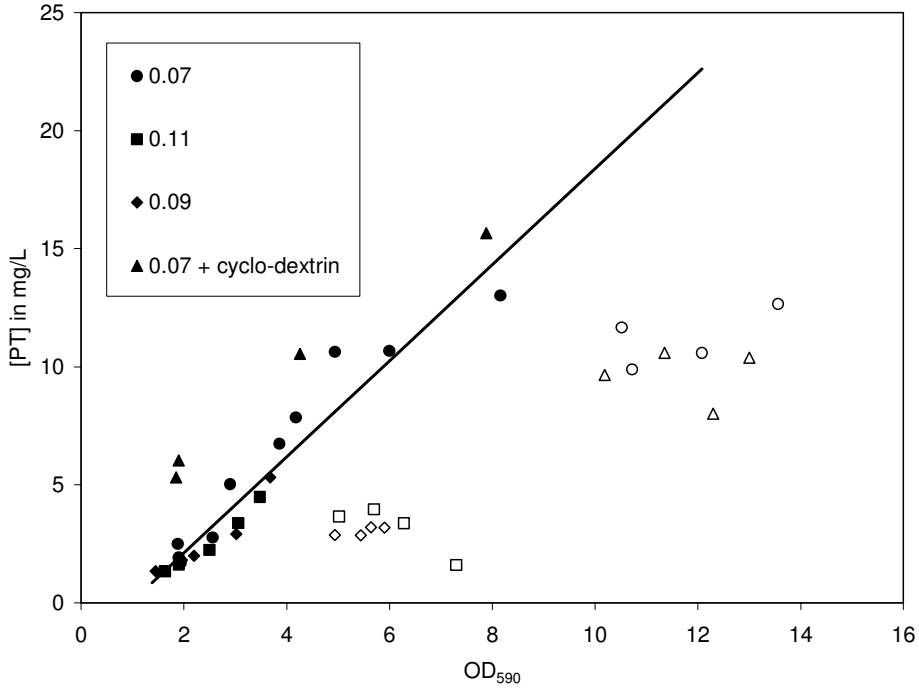

Figure 5. PT content of culture liquid plotted against the Optical Density of the cultures. The closed symbols indicate where the PT content was increasing during the culture, while the open symbols of the same culture indicate where growth continued, but with no further PT increase. The open symbols were not included in calculating the average PT content per unit Optical Density at 590 nm. The culture with cyclo-dextrin was excluded from these calculations.

The specific productivity of PT production during the feed phase was calculated using all data points where the [PT] and the OD were measured. The culture with cyclo dextrin was not included because of the higher PT concentrations during the batch phase. Since PT production ceased before the end of growth, only the data points before PT production ceased were taken into account, i.e. the data points with closed symbols. As such, the specific product yield was 2.0 ± 0.23 mg PT/OD unit, indicated in Figure 5 as a trend line with a slope of 2.0 mg PT/OD_{590} unit. Since for these fed-batch cultures the OD_{590} correlates well with dry weight (no data shown), the specific productivity of PT was approximately 4.0 mg PT/g biomass. This productivity compares quite favorably with the productivity at the end of the batch phase of 2.2 mg PT/g biomass. For all cultures the level of PT remained fairly constant after PT production ceased. The productivity for the entire culture, i.e. until growth stopped, was 1.9± 0.09 mg PT/g biomass for the cultures receiving the feed rate of 0.07 h^{-1}.

4. Concluding remarks

The present study showed that it is possible to increase the amount of *B. pertussis* biomass in bioreactor cultures, up to 7 times the density of batch cultures, by employing relatively straightforward lactate and glutamate feed media and feed strategies. Feed rates enabling growth rates of 0.09 h^{-1} and higher lead to accumulation of substrates and decreased biomass yields. PHB formation occurs when lactate is detectable in the culture broth while glutamate is not. When both lactate and glutamate are detectable, PHB is broken down again. Substrate levels in the millimolar range correlate with increased free fatty acid formation and eventual cessation of growth. However, free fatty acid formation is not the reason growth stops; free fatty acid formation appears to be a result of rather than the cause for the decreased and eventual cessation of growth. The reason why the organism cannot maintain high growth rates could be the addition of Na^+ to the bioreactor due to the Na-glutamate feed which significantly lowers the maximal growth rate. Adding glutamic acid as a suspension rather than a solution of Na-glutamate could circumvent this problem. The reason why growth stops entirely is not known at this point.

PT production is growth-associated for a major part of the fed batch culture, with a productivity of 4 mg PT.g^{-1} biomass. However, PT production stops before growth does, leading to a final PT yield of 2 mg PT.g^{-1} biomass. Given the fact that all amino acids can be produced in sufficient amounts, the cessation of PT production may be a consequence of regulatory mechanisms rather than a feed or metabolism related phenomenon. The fact that cultures with high growth rates stop producing PT relatively early in the cultivation suggests that the factor responsible for this regulation is produced more rapidly at higher growth rates. Although these regulatory aspects are a problem for growing laboratory or wild type strains, genetic modification enables deletion of any regulatory sequences, which enable high cell density and high PT production.

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Everyone is entitled to their own opinion, but not their own facts.

--**Daniel Patrick Moynihan**

CHAPTER 6

Whooping cough vaccines: a public health and producer's perspective

Summary

Despite the high vaccination coverage in most of the Western world, the incidence of whooping cough has been increasing in all age groups for the last 2 decades. The global rise in whooping cough cases did not lessen after the introduction of acellular vaccines a decade ago, or the introduction of booster vaccinations in toddlers. Currently registered vaccines are not well equipped to overcome the rising incidence of whooping cough for a number of reasons. Therefore, there is a definite need for an improved whooping cough vaccine. Given the numerous possibilities to improve existing vaccines, this paper sought to limit the number of possibilities by rationally selecting the characteristics of an improved vaccine. An improved whooping cough vaccine should (1) enable all age groups to be vaccinated with *B. pertussis* circulating strain antigens, (2) protect against whooping cough induced by *B. parapertussis*, (3) enable infants to be vaccinated earlier, (4) cause minimal adverse events after repeated vaccination, and (5) protect longer than currently registered vaccines. A number of scenario's and corresponding vaccine compositions that comply with (part of) these characteristics were examined, using time to market, costs and risks as constraints. The most likely candidates to fulfill all characteristics are oral or intranasal vaccines consisting of inactivated whole *B. pertussis* cells, given the fact that an oral vaccine has already shown proof of protection in a phase III study without adverse events, and that an intra-nasal vaccine has shown proof of concept in a phase I study. Live attenuated vaccines fulfill many of the 5 characteristics as well, but will most likely take longer to reach the market. At this point it is not clear if a *B. parapertussis* component should also be included in an improved whooping cough vaccine, and what the cost-benefit ratio would be.

key words: improved whooping cough vaccine, vaccination schedule, oral, nasal vaccination

1. Introduction

Whooping cough was a common cause of infant mortality before a vaccine was available. The disease derives its name from the characteristic, severe hacking cough followed by an intake of breath that sounds like 'whoop'. The coughing fits in young babies and children can lead to vomiting, turning blue due to insufficient breathing (cyanosis) and even temporary cessation of breathing altogether (apnoea). Due to complications of these symptoms, mortality in unvaccinated individuals was and still is not uncommon. This quite contagious disease is transmitted by aerosols that are formed when a person with whooping cough talks, sneezes or coughs. Although the severe coughing easily lasts more than a month, an individual is most contagious during the second to fourth week after being infected.

Whooping cough is caused by *Bordetella pertussis* and *Bordetella parapertussis*. Despite the fact that *B. parapertussis* can cause severe disease, all whooping cough vaccines are based on *B. pertussis*. The first generation vaccines consisted of inactivated whole cells and were first applied in the 1950's. These whole cell vaccines (WCV) virtually eliminated pertussis associated infant deaths. The disease itself was marginalised and pertussis in vaccinated individuals mostly leads to only a mild form of the disease. The success of WCV's is at least partly due to the simple and inexpensive production process; *B. pertussis* is cultivated, centrifuged, resuspended and inactivated. In contrast, to produce these suspensions consistently is far more complicated (Thalen, 2008). Since the antigenic composition of the pertussis cells, i.e. the vaccine composition, is formed during the cultivation step, the reproducibility of this step is crucial. Unfortunately, often poorly defined components like casein-hydrolysates and/or yeast extract are used in *B. pertussis* media, which inherently vary from batch to batch. This variation in turn causes batch to batch variation in the cultivation, which leads to batch to batch variation in the potency of the vaccine. Indeed, several authors (Csizer, 1977, Baraff, 1984, Steinhoff, 1995) found large differences in potency between producers, as well as between batches of the same producer.

After whooping cough disappeared as a common disease, parents born after the mass vaccinations in the 50's had no experience with the severity of the disease in unvaccinated infants. Consequently, public concerns shifted from fear of the disease to fear of the side-effects of the vaccine. Since the inactivated whole cells in cellular *B. pertussis* vaccines contain lipo-polysaccharide (LPS), vaccination frequently caused fever. While the symptoms of fever are mostly innocent, the rare and more severe adverse effects such as convulsions and hypotonic hyporesponsive episodes[1]) (HHE) were perceived as quite alarming, even though

these always passed with complete recovery. Especially the convulsions and HHE were responsible for the unfounded but persistent perception that whole cell pertussis vaccination can cause neurological symptoms. Invariably, the public reaction to real or perceived vaccination risks results in reduced vaccination coverage. The devastating consequences that perceived vaccination risks can have are discussed in Intermezzo I.

Due to safety concerns, a number of countries decided in the 70's and 80's that it was safer to stop using WCV's and wait until a safer whooping cough vaccine was developed. In the 80's, Japan was the first country to develop and introduce whooping cough vaccines that consisted of *B. pertussis'* outer [1]membrane extracts, mixed with semi-purified culture supernatants. Typically, these second generation so-called ACellular Vaccines (ACV) contain Pertussis Toxin (PT), Filamentous Heme-Agglutinin (FHA), Pertactin (PRN), Fimbriae (FIM) as well as some LPS. Since the amount of these components could be quantified, the production consistency of the ACV's was generally better than that of WCV's. The adverse events of the ACV's were less than those of the cellular vaccines. Thus, fever after vaccination was significantly reduced, while the rare adverse events such as the convulsions and HHE occurred even less frequently than after WCV vaccination. The amount of the virulence factors per dose was 2 to 10 fold higher than the amount of virulence factors present in one dose of whole cell vaccine. Therefore, the increase thus needed in fermentation and post harvest processing capacity lead to an estimated 5 fold cost price increase per dose as compared to that of a WCV's. These second generation vaccines are still in use today in the form of the Biken and Takeda vaccines, which are registered in Japan, USA and Canada.

With the increasing knowledge of *B. pertussis'* pathogenesis and the use of molecular biology tools, the third generation whooping cough vaccines was developed and tested in the 90's. These ACV's consist of 1 to 5 highly purified antigens, most of which are chemically inactivated. The use of highly purified antigens offers the possibility to greatly increase the consistency of production. Due to the extensive processing and purification processes involved, the cost price of these ACV's is at least 10 to 25-fold higher than that of a cellular vaccine. During the last decade of the previous century, more than 20 different ACV's were tested in clinical trials. Now, almost a decade later, only 3 of these newly developed acellular vaccines are registered throughout the western world. They are applied routinely and have a safety profile that is similar to that of the second generation vaccines.

[1] These term and abbreviations are explained in the glossary.

Although ACV's in general have a better safety profile than most WCV's, on a number of other points the merits of ACV's are less than satisfactory. A number of observations from very diverse fields indicate that the currently registered vaccines, both cellular and acellular, are not well equipped to take on the challenges of the coming decades. For example, the antigens used in the currently registered acellular vaccines do not match well with the antigens found in *B. pertussis* strains isolated from patients (Mooi, 1998, 2007). Also, *B. parapertussis* is isolated frequently from patients with whooping cough and the acellular vaccines do not protect against *B. parapertussis* (Mastrantonio, 1997, Stehr, 1998). The adverse events of acellular vaccines during the first series of vaccinations increase with each additional dose of vaccine (Rennels, 2003). Therefore, it remains to be seen how well currently registered low dose booster vaccines can be used to vaccinate adolescents and adults that have received the 4 to 5 doses during their first years of life. Nevertheless, there is a need for pertussis vaccination of adolescents and adults, since whooping cough in these groups has shown a steady increase over the last decades. This increases the chances of unvaccinated or incompletely vaccinated infants in the age group 0 - 5 months to be infected by an adult. A most disturbing observation is that in a number of recent pertussis outbreaks, 10 to 30% of the infants and/or toddlers were fully vaccinated with ACV's (review Celentano, 2005). A more detailed discussion of these and other key issues with currently registered whooping cough vaccines is given in Intermezzo II.

The global increasing incidence of whooping cough, the threat of *B. parapertussis* induced whooping cough, the occurrence of whooping cough outbreaks in fully vaccinated populations, and the fact that the 0 - 5 months age group is insufficiently protected, underline that there is a need for an improved whooping cough vaccine. This paper addresses the question what such a vaccine should look like from a theoretical and a pragmatic perspective. Firstly, the performance parameters of such an improved vaccine are defined in terms of protection and safety. For every performance parameter various ambition levels are defined. For example, a low ambition level for the parameter protection corresponds with preventing infant mortality, while a high ambition level corresponds with protection against transmission of whooping cough. Ideally, only vaccines with a high ambition level would be considered acceptable, but reality imposes restrictions in terms of the time and costs involved in developing, carrying out clinical trials with, and registering such an improved whooping cough vaccine. Therefore this paper also addresses the consequences of the various ambition levels for an improved whooping cough vaccine, using time to market, costs and risks as constraints. This way, vaccine development strategies are identified that are most viable from a commercial point of view, and therefore most likely to lead to a successful product.

2. Improved whooping cough vaccine: establishing performance parameters and their ambition levels

A pre-requisite for an improved whooping cough vaccine to be accepted by the general public is that it equals or preferably exceeds current ambition levels for all performance parameters of currently registered vaccines. In this paragraph a number of performance parameters are identified by which to judge an improved vaccine. For each of these performance parameters the various ambition levels will be addressed and quantified if possible. A summary of the details of these performance parameters is given in Table 1 for protection and Table 2 for safety. The various ambition levels of the performance parameters described below do not just attempt to protect infants and toddlers from pertussis, but adolescents and adults as well, since whooping cough can no longer be seen as a childhood disease only.

2.1 Prevention of whooping cough

Current ambition level (Table 1, ambition level 1.1-1.3). Most current whooping cough vaccination programs aim at preventing infant mortality and hospitalization of infants and toddlers from *B. pertussis* induced whooping cough. Since many infants catch whooping cough from their parents or grandparents, the ability to vaccinate adults is essential, since vaccination will most likely prevent infection and transmission to infants. In addition, whooping cough in adults or adolescents causes considerable economic damage. Recently, a number of low dose booster acellular vaccines (aCV) for adolescents or adults was registered in a number of countries. This means that in principle all age groups can be protected from mild to severe *B. pertussis* induced whooping cough, although it remains to be seen how well this vaccine is tolerated in adults that have received the primary series of the same ACV's, as discussed further under paragraph 2.3.

Next ambition level: Incorporation of antigens from circulating strains (Table 1, ambition level 1.4). A flaw of all *B. pertussis* derived whooping cough vaccines, cellular and acellular alike, is that *B. pertussis* strains currently isolated from patients produce immunologically different antigens than the strains that are used to produce whooping cough vaccines (Intermezzo II; Antigenic variation). Therefore, the next ambition level consists of a whooping cough vaccine that immunogenically matches currently circulating strains (csACV), and that can be used for infants, toddlers as well as for adolescents and adults.

Next ambition level: Protection against B. parapertussis (Table 1, ambition level 1.5). A second inadequacy of all currently registered ACV's is that no vaccine significantly protects against whooping cough caused by *B. parapertussis*. WCV's offer partial protection against *B. parapertussis* in animal models (David, 2004), and may also provide partial protection against *B. parapertussis* in humans. The clinical symptoms of whooping cough caused by *B. parapertussis* are the same as those caused by *B. pertussis*, and often equally severe. Therefore, an improved whooping cough vaccine should not only protect a population against *B. pertussis*, but against *B. parapertussis* as well.

Final ambition level: prevention of colonization and transmission. The next ambition level can be defined as an improved vaccine that prevents colonization and transmission of whooping cough. However, due to the fact that there is insufficient data available regarding this subject, it is not included in this paper.

2.2 Vaccination schedule

Current ambition level: age at completion of primary series (Table 1, ambition level 2.1). All registered vaccines against whooping cough can be administered to infants from the age of 2 to 3 months onwards. Therefore, during those first two to three months an infant is completely unprotected against whooping cough other than by maternal antibodies if any are present. After the first vaccination, generally 2 more vaccinations follow, each one month after the other. After the infant's 3rd vaccination, i.e. at the age of 5 to 6 months, protection against pertussis can be considered complete, although most countries administer also a booster vaccination when the infant is approximately 1 year old.

Current ambition level: booster vaccinations every 4 - 8 years (Table 1, ambition level 2.2). After the booster vaccination at age 1, a second booster is generally applied at age 4. The number of years that an individual is protected against whooping cough after the last booster vaccination currently varies from 4 to 8 years (Wirsing von Konig, 1998). Assuming an average duration of protection of 6 years, an individual would need approximately 17 vaccinations against whooping cough to be protected for life.

Next ambition level: early completion of primary series (Table 1, ambition level 2.3). In the USA, as in most countries, most of the hospitalisations due to whooping cough as well as rare cases of infant mortality occur in the age-group of 0 to 5 months (Cortese, 2008). This 0 to 5 months period corresponds with the period in which infants receive the primary vaccination series and are therefore only partially protected against whooping cough. Therefore, ideally, infants should

receive the first vaccination weeks rather than months after birth. Also, the time to complete the primary vaccination series should be reduced to minimize the period during which an infant is only partially protected. The problem is that current injectable ACV's cannot be applied at an earlier age due to the immaturity of the infant's immune system. However, since *B. pertussis* and *B. parapertussis* are mucosal pathogens, it makes sense to administer the vaccine orally or intranasally rather than parenterally. Both the oral and intranasal mucosa can generate a mucosal immune response when triggered, even shortly after birth (Baumann, 1985, Mascart, 2003).

Next ambition level: booster vaccination every 10 -15 years (Table 1, ambition level 2.4). The longest protection against whooping cough is conferred by a natural infection, which is estimated to protect for 10 to 15 years (Wirsing von Konig, 1998). As such, the duration of protection of an improved whooping cough vaccine should preferably be similar to that induced by natural infection, i.e. 10 years or more. Thus, assuming that adults would need to be vaccinated every 10 years, approximately 7 vaccinations are needed during an adult life, next to the 4 to 5 vaccinations received during childhood and adolescence. In total an improved whooping cough vaccine would have to be applied approximately 11 times during a lifetime.

Table 1. Performance parameters related to protection of individuals against whooping cough, the ambition level increasing in descending order. The current ambition level is shown in ***bold italics***.

performance parameter	ambition level	ambition level associated vaccine composition	remarks
1. prevention of whooping cough induced	***1.1 hospitalization and mortality of infants***	a - inactivated *B. pertussis* cells (WCV) b - *B. pertussis* purified antigens (ACV)	- mortality is quite rare after a complete set of vaccinations has been received - in whooping cough outbreaks, hospitalization (<15%) is seen in completely vaccinated individuals after vaccination with ACV's
	1.2 hospitalization of toddlers	- *B. pertussis* purified antigens (ACV)	- adverse events of WCV's are too high for toddlers, therefore only ACV's are considered
	1.3 mild to severe symptoms in all age groups	a - *B. pertussis* purified antigens for toddlers and infants (ACV) b - low dose *B. pertussis* purified antigens adolescents and adults (aCV)	- complete recovery of whooping cough in toddlers, adolescents and adults can take weeks to months - a number of countries where low content boosters were recently registered, adolescent and/or adult vaccination is recommended
	1.4 mild to severe symptoms in all age groups against circulating *B. pertussis* strains	- *B. pertussis* purified antigens that match ***c***irculating ***s***trains (csACV, csaCV)	- the superiority of using an cs-a/ACV, may only become apparent when immunity starts to wane, i.e. longer duration of protection - current aCV's could be unsuitable for adults that have received the full series of childhood ACV vaccination.
	1.5 mild to severe symptoms in all age groups against circulating *B. pertussis* and *B. parapertussis* strains	a - *B. pertussis* and *B. parapertussis* purified antigens that match circulating strains	- no known vaccine development is being carried out to develop an acellular *B. parapertussis* vaccine
		b - *B. pertussis* purified antigens and *B. parapertussis* cells	- combination whole cell vaccines of *B. pertussis* and *B. parapertussis* cells developed and tested in Czechoslovakia and Russia, showed efficacy against both bacteria while the safety was comparable to DPT vaccination
2. Vaccination schedule	**2.1 completion of primary series 4 - 5 months**	- injectable WCV or ACV	- first vaccination at 2 - 3 months of age, completion at 4 or 5 months of age, hospitalization is highest in this age group
	2.2 booster vaccination every 4 - 8 years	- injectable ACV	- current duration of protection induced by ACV's means ±17 vaccinations during adult life
	2.3 completion of primary series 1 - 5 weeks	a - oral inactivated whole cell vaccine b - intranasal inactivated whole cell vaccine	- the infant mucosal immune system is fully functional directly after birth and can be triggered either orally or intranasally with inactivated whole cells
	2.4 booster vaccination every 10 - 15 years	a - intranasal or oral inactivated whole cell vaccine	- no long term data on duration of protection are available for oral or intranasal vaccines
		b - intranasal or oral live attenuated vaccine	- live attenuated vaccine will probably only require 1 dose in the primary series and has shown protection against *B. parapertussis* in a mouse model

2.3 Adverse events

Current ambition level (Table 2, ambition level 1.1 to 1.6, 2.1 and 2.2). Table 2 shows an overview of the major categories of adverse events, which occur after WCV and/or ACV whooping cough vaccinations. The effects can be categorized as local, i.e. events close to the injection side, or systemic events, which affect the vaccinee in general. Systemic events are perceived as more serious. Some reactions are inherent to alum-adjuvated injectable vaccines such as some degree of fever, various reactions at and around the injection site, as well as vomiting in some cases. As such, currently registered WCV's, ACV's and aCV's also cause a number of adverse effects, mostly harmless and frequent (Table 2, ambition level 1.4 - 1.6), incidentally more severe and rare (Table 2, ambition level 1.1 - 1.3).

Next ambition level: no observable adverse events (Table 2, ambition level 1.7 and 2.3). For currently registered ACV's, the infrequent adverse events such as swelling of an entire limb or persistent nodule formation (Table 2, ambition level 1.4 and 2.1, respectively) are considered as tolerable, since the general public compares these to the adverse events of a whole cell vaccine. In a number of years, as the comparison with the whole cell vaccine has faded, these side effects may no longer be considered tolerable. To reduce these side effects, 2 low dose acellular vaccines or aCV's have been developed for adult and adolescent vaccination. These contain less than 30% of the amount of antigens present in the ACV's. Initial studies with aCV's as 6^{th} dose in adolescents caused more pain than the 5^{th} dose (Zepp, 2006), so it remains to be seen how well currently registered aCV's are tolerated after repeated vaccination by adults that were vaccinated with ACV's as the primary series. Therefore, an improved whooping cough vaccine should enable repeated vaccination of infants, toddlers, adolescents and adults without accumulating local or systemic adverse events (Table 2, ambition level 1.7 and 2.3, respectively) due to repeated vaccination. Since adverse events are a direct consequence of the vaccine formulation and the route of administration, these are not discussed separately but together with the individual potential vaccine formulations and routes of administration.

Table 2. Performance parameters related to the safety profile of current and improved whooping cough vaccines, the ambition level increasing in descending order. The current ambition level is shown in ***bold italics***. (i.d.)

performance parameter	ambition level	remarks
1. avoid systemic adverse events	***1.1 hypotonic-hyporesponsive episode & convulsions***	occurs in WCV vaccinees (<1:100.000), very rarely in ACV's
	1.2 fever (>40 °C)	occurs in <0.2% of WCV and in <0.1% of ACV vaccinees
	1.3 vomiting	occurs in <1% in both ACV and WCV vaccinees
	1.4 swelling of entire limb	occurs in 1 - 2% of ACV vaccinees, not in WCV vaccinees, increases after booster vaccination
	1.5 persistent screaming	occurs in 4 - 12% in both ACV and WCV vaccinees
	1.6 fever (>38 °C)	occurs in 5 - 40% of WCV vaccinees, and 0.5 - 5% of ACV vaccinees
	1.7 no observable adverse events	virtually all injectable vaccines give rise to some systemic adverse events
2. local adverse events	***2.1 persistent nodule formation***	occurs in 2 - 6% of ACV vaccinees, not after WCV vaccination
	2.2 injection site reactions >50mm	4 - 12% of WCV and ACV vaccinees
	2.3 no observable adverse events	virtually all injectable vaccines give rise to some local adverse events

Note that there are large differences in adverse events from producer to producer of WCV's, which are much larger than the differences in adverse events between ACV's (review Jefferson, 2003).

2.4 Profile of an improved whooping cough vaccine

From the paragraphs above it is clear which ambition levels of the current whooping cough vaccines need to be increased in order to prevent whooping cough in infants, adolescents and adults. Following the sequence of ambition levels in Table 1, firstly, the antigenic mismatch between vaccine antigens and the antigens in strains isolated from whooping cough patients (Intermezzo II, antigenic variation) needs to be corrected by incorporating the circulating antigens into the improved vaccine. The protection against whooping cough caused by *B. parapertussis* is the second issue that needs to be addressed by an improved whooping cough vaccine. Third, there is a need for a vaccine that enables a vaccination schedule that protects infants earlier, thereby preventing the typical symptoms of whooping cough that can be life-threatening in the 0 to 5 months age group. Aside from the protection induced by the improved vaccine, the adverse events also need to be addressed, given the fact that these vaccines will need to be applied repeatedly in life, not just during childhood, but also in adolescent and adult life. Especially the increase in side effects with each consecutive dose is incompatible with vaccination throughout life. Last on the list is the duration of

protection, which would ideally be as long as a natural infection, i.e. 10 - 15 years, but preferably longer than the 4 to 8 years protection current vaccines offer. In summary the five main characteristics of an improved whooping cough vaccine are:

I - enables all age groups to be vaccinated with *B. pertussis* circulating strain antigens
II - protects against whooping cough induced by *B. parapertussis*
III - enables earlier protection of infants against whooping cough
IV - causes minimal adverse events after repeated vaccination
V - protects longer than currently registered vaccines

3. Development of improved vaccines

3.1 Constraints of commercial vaccine development: ambition level versus development time, costs and risks

The typical activities and their duration prior to registering a vaccine entail the development of (I) a suitable vaccine composition, as well as the Quality Control (QC) assays to measure the quality of that vaccine composition (2 - 5 years), (II) a Good Manufacturing Practice (GMP) production process for intermediate to large scale production of that vaccine (1 - 2 years), and (III) the phase I to III clinical trials to test the safety and efficacy of the vaccine composition in humans (>4 years). Only after these three phases are completed successfully can a vaccine be submitted for registration and produced for commercial use. Typically, each consecutive phase requires an order of magnitude more costs for it to be completed.

Developing the vaccine formulation and the production process generally takes half of the typical decade needed to register a vaccine. The duration of this phase can be influenced significantly by a number of factors. The development time for an entirely new vaccine formulation is significantly longer than for a vaccine formulation that is similar to existing, registered vaccines. Also, the existing expertise of a manufacturer plays a role in the development timeline. For example, an existing ACV manufacturer will develop an improved ACV more quickly than a manufacturer that only has experience in WCV manufacturing.

Clinical trials are difficult to shorten in terms of duration. However, the costs of a clinical trial can be reduced by simplifying it. For example, the efficacy of a stand alone whooping cough vaccine is easier to determine than if the whooping cough vaccine is part of a combination vaccine. Likewise, a single booster vaccination in

adolescents and adults is easier to evaluate than the three consecutive vaccinations typically required to vaccinate infants. Also, the definition of the aim of the clinical trial has an impact on the statistically required number of participants needed to complete the trial. For example, if the aim is to show that the vaccine is better than an existing vaccine, more participants are needed than if the criterion is 'similar or better'. Intermezzo III describes a number of specific difficulties associated with whooping cough clinical trials.

Risk mitigation is imperative while developing a vaccine, given the time and costs involved. Chances that a vaccine may fail are significant, and any reduction in risk will make it more attractive for a manufacturer to commit to a development program. Since typically most of the costs are made in the clinical trial phase, this is where risk mitigation has the highest priority. Therefore, any proof of concept or protection in humans of the type of product to be developed greatly reduces the risk of failure in late stage development. Developing a completely new vaccine is therefore often avoided.

In the following paragraphs, the first three characteristics of the profile of an improved whooping cough vaccine described in paragraph 2.4 are translated into vaccine compositions, which are evaluated using development time, costs and risks as constraints. The remaining two characteristics, adverse events and the duration of protection, are largely a consequence of the vaccine composition chosen, and will be discussed together with the vaccine composition.

3.3 Incorporation of *B. pertussis* circulating strain antigens

The development of vaccines containing the antigens of circulating strains (Table 1, ambition level 1.4) requires that the appropriate genes from the *B. pertussis* strains are isolated and incorporated in commonly used vaccine production strains. The resulting recombinant strains can either be used for the production of an ACV or a WCV. Either way, the existing production processes for the existing vaccines will probably not need to be modified, since the biochemical differences between the antigens of the circulating and current vaccine strains are marginal. Some of the QC assays may need to be modified, but the overall time needed to develop such a vaccine will be less than 5 years. While the clinical trials focussing on safety and efficacy of such a vaccine still need to be carried out, from a regulatory point of view it will be easier to register such a vaccine, since it is quite similar to vaccines that are already registered. The risk of developing this type of vaccine is not so much in the potential vaccine failure, but more in size and the duration of the clinical trials to show that a vaccine with the divergent antigens protects significantly better or longer than vaccines with the current composition.

Therefore, the clinical end point should probably be 'similar or better protection' in order to avoid unnecessarily large and long clinical trials. In short, an existing manufacturer should be able to register a vaccine that corrects the mismatch between vaccine and circulating strains in less than the typical decade.

3.4 Protection against *B. parapertussis* induced whooping cough

Developing a *B. parapertussis* component to be included into an improved whooping cough vaccine is a challenge (Table 1, ambition level 1.5). Although *B. parapertussis* shares many of the functional virulence factors with *B. pertussis*, the immunological cross-reactivity and thus cross-protection is limited (Khelef, 1993, David, 2004). Assuming the same antigens are important in protection, it is possible to choose a number of *B. parapertussis* antigens, and evaluate these in available mouse models (David, 2004, Watanabe & Nagai, 2003). Since no acellular vaccine has been developed so far against *B. parapertussis*, the development phase is difficult to shorten, although the production process and the QC assays will probably be similar to those of *B. pertussis* and therefore be fairly straightforward to develop. Once this *B. parapertussis* acellular vaccine has been formulated, it can be incorporated into an existing or improved *B. pertussis* ACV and tested in clinical trials. The clinical trial will be more complicated than a 'normal' whooping cough clinical trial, since every whooping cough case that occurs in the trial has to be analysed whether it was caused by *B. pertussis* or by *B. parapertussis*. Since *B. parapertussis* generally occurs 2 to 3 fold less frequently than *B. pertussis* (review Watanabe & Nagai, 2004), the statistically required participants in the trial will have to be at least 2 to 3 fold higher than for a *B. pertussis* whooping cough clinical trial. The risks of developing an acellular *B. parapertussis* vaccine therefore are the costs involved in the size and duration of the clinical trials, while there is no proof of concept in humans for a *B. parapertussis* acellular vaccine in combination with a *B. pertussis* ACV. The total time to market of such a vaccine will probably take longer than a decade. Therefore, while the approach is valid from a scientific point of view, from a commercial point of view it is unattractive to undertake such a long and costly development programme, without a reasonable degree of certainty that an acellular vaccine against *B. parapertussis* will work, or what the side effects will be.

There may be another way to achieve the incorporation of a *B. parapertussis* component in an ACV, that has shown proof of protection in humans. In Czechoslovakia (Buriănová-Vysoká, 1970) and Russia (Demina, 1969) whole-cell *B. parapertussis* vaccines were developed that were included in routine Diphtheria

Tetanus and Pertussis (DTP) combination vaccines, to form Diphtheria Tetanus Pertussis Parapertussis (DTPP) combination vaccines. These cellular *B. parapertussis* vaccines were produced using the same production process as used for *B. pertussis* cellular vaccines, only the number of bacteria per dose was 25% of the number of *B. pertussis* organisms in the DPT vaccine. The DTP and DTPP vaccines were tested in large clinical trials. The efficacy of the DTP and DTPP vaccines against *B. pertussis* induced whooping cough was comparable, but the DTPP vaccine protected significantly better against *B. parapertussis* induced whooping cough. The safety profile of the DTPP vaccine was comparable with the DTP vaccine of the same producer (Burián, 1970). This study showed that the DTPP vaccine caused similar adverse events as the DTP vaccine produced by the same manufacturer, i.e. the *B. parapertussis* component did not increase the adverse events caused by the *B. pertussis* component of the DTPP vaccine. Since the number of *B. parapertussis* cells used in the *B. parapertussis* component is quite low, it is conceivable to incorporate a cellular *B. parapertussis* vaccine into an existing ACV. In this way, existing ACV and whole cell *B. parapertussis* vaccine compositions, production processes and QC assays can be used to test the combination vaccine in phase I through III clinical trials. The clinical trials will still be long and costly for the same reasons given in the previous paragraph. However, since whole-cell *B. parapertussis* vaccines have already demonstrated proof of protection in infants, the risks of developing such a vaccine are less. The real risk of this development program is not so much in the product as in the public acceptance of the product. It will be difficult to defend incorporating a cellular *B. parapertussis* component into an acellular *B. pertussis* vaccine that took 2 decades to develop in order to replace the cellular *B. pertussis* vaccine.

3.5 Earlier protection of infants: nasal, oral and live vaccines

Since injectable ACV's cannot be administered effectively prior to 2 months (Mascart, 2003), the only way to complete the primary series of vaccination earlier is to use oral or intranasal administrated vaccines (Table 1, ambition level 2.3, vaccine composition a). Baumann (1985) showed that a stand alone oral inactivated *B. pertussis* vaccine without adjuvant was effective in a clinical trial with over 20.000 infants. This product was given orally on day 2, 3, 4 and 5 after birth, with a booster after 6 weeks. Since the booster administered after 6 weeks did not increase mucosal antibody levels further, it could be argued that vaccination was complete 5 days after birth. The protection against whooping cough during the first year was at least as good as the same inactivated vaccine administered parenterally at 3, 4 and 5 months. There are two main advantages of

an oral inactivated *B. pertussis* suspension. For one, this vaccination schedule limits the time that infants are not or partially protected from 4 to 5 months down to one week. The other major advantage of this approach is that the vaccine was tolerated without ***any*** reported adverse effects, which is to be expected from an inactivated, oral, non-adjuvated vaccine. The fact that this is a stand alone vaccine also makes clinical trials less costly to carry out.

The reason the vaccine was not developed further may have been related to the dose applied: 10^{12} bacteria per dose rather than the 10^{10} bacteria per dose for injectable vaccines, i.e. a factor 100 more biomass. At the time the study was carried out, an 100-fold increase in production capacity would have had a considerable impact on commercial production. Since then, cultivation techniques have improved, yielding not only more biomass (Licari 1991, Frohlich 1995, Thalen 2006), but also more immunogenic biomass (Thalen, 2008). The investment for this type of vaccine is primarily in the phase I to III clinical trials, since the production processes for this type of vaccine are available. However, the animal models to test these vaccines will need to be modified. The fact that phase III studies have been carried out with this type of vaccine with excellent results also limits the investment risk. Therefore, an oral, stand-alone whooping cough vaccine could probably be on the market in less than the average of a decade.

More recently, Berstad (2000) has used intranasal vaccination in 6 adult volunteers using inactivated *B. pertussis* cells (Table 1, ambition level 2.3, vaccine composition b). Each dose consisted of approximately 10^{10} bacteria, and was given 4 times, with one week in-between doses. All volunteers raised specific mucosal IgA antibodies, while 3 of the vaccinees also developed serum IgA and IgG titers. This work showed that significant immune responses can be generated in humans with a dose of *B. pertussis* cells that is comparable to the amount of cells in an injectable cellular *B. pertussis* vaccine. While this work needs to be taken further before it can be assessed in phase II or III trials, it is safe to assume that the adverse events associated with an intranasal whole cell vaccine will be negligible compared to an injectable vaccine. Also for this approach production processes are available, so that the time to register such a vaccine is primarily determined by the duration of the phase I to III clinical trials, which leads to the similar timelines as the oral vaccine. The main difference is that the intranasal vaccine has not been tested in infants yet, so the risk of investment is higher.

Next to using inactivated *B. pertussis* whole cells for intranasal vaccination, Mielcarek (2006) has developed an intranasal vaccine based on a live, attenuated strain of *B. pertussis*, which was evaluated in a mouse model (Table 1, ambition level 2.4, vaccine composition b). The advantages of a live attenuated vaccine are that the immunity it induces is even more similar to a natural infection as

compared to using inactivated cells. Also, in mice one vaccination was sufficient to confer protection instead of having to vaccinate 3 to 4 times. Since the immune system of unvaccinated new born infants is triggered appropriately by a *B. pertussis* infection (Mascart, 2003), it is reasonable to assume that an attenuated strain can do the same. Therefore, a live attenuated strain can probably also be applied relatively early, i.e. weeks after birth rather than months. Interestingly, this attenuated strain also protected mice against *B. parapertussis* infections. Since this type of vaccine has not been tested in humans or primates, it is difficult to predict which adverse events will be associated with an attenuated strain, since this largely depends on the type of attenuation chosen, and how this influences the organisms' ability to cause disease. Potentially, an attenuated vaccine could protect as long against *B. pertussis* as a natural infection. The risk of this approach is, as with using any attenuated vaccine, that there will always be immuno-compromised individuals that cannot clear the attenuated organism from their system, and may develop symptoms of the disease. An additional risk of this approach is that the first clinical trials will be subjected to rigorous safety precautions to stop the attenuated strain from spreading to the environment until it is established that the strain is harmless. Whether justified or not, this will add significantly to the costs of the clinical trials. Therefore, this quite promising approach currently has more risks associated with it than using inactivated cells.

4. Improved whooping cough vaccines: ways forward

Despite the high vaccination coverage in most of the western world, the incidence of whooping cough has been increasing in all age groups for the last 2 decades. The global rise in whooping cough cases did not lessen after the introduction of acellular vaccines a decade ago. Although vaccination of toddlers with ACV's did lower the incidence of whooping cough in toddlers, the incidence in adults and adolescents increased. The immediate action that can be taken to better protect infants from contracting whooping cough is to vaccinate adolescents and adults with recently registered acellular booster vaccines so as to decrease the chances of infant infection. However, once the infants of today become parents themselves, it remains to be seen how well they will tolerate currently registered acellular booster vaccines. Nevertheless, vaccinating adolescents and adults that have not been vaccinated with acellular vaccines as infants does buy time to develop a better whooping cough vaccine.

When examining the various vaccine tentative compositions in the previous chapter, it is clear that an oral stand alone *B. pertussis* vaccine fulfills most of the criteria of the profile of an improved whooping cough vaccine. The trial by Bauman (1985) showed proof of protection of a stand alone oral whole cell *B. pertussis* vaccination within a week after birth. It is unlikely that *B. pertussis* genetically

modified to include circulating strain antigens would be less effective. Next to the early protection, the absence of adverse events associated with oral delivery may be the biggest advantage of this vaccine, since this means that it can probably be administered repeatedly to toddlers, adolescents and adults alike without adverse events. If vaccination can be carried out without adverse events, the duration of protection becomes less of an issue. At this point no long term protection data are available for oral whooping cough vaccines.

The development that needs to be done for an oral vaccine is to re-establish the dose in the phase II clinical trials, checking if the 10^{12} bacteria per dose is really necessary with current media and cultivation technologies. The intranasal formulation used by Berstad (2000) requires only 1% of the bacterial mass of the oral vaccine Baumann (1985) used, which means that the administration route has quite an impact on vaccine costs. It could be beneficial to test an oral vaccine in diluted form, administered as an intranasal vaccine in one phase II clinical trial. Including an extra study group in a phase II clinical trial is not prohibitively expensive, while the benefits are obvious. Also, it may turn out that an oral formulation is more suitable for infants, while a booster vaccination for toddlers, adolescents and adults could also be supplied as an intranasal formulation. The relatively short time to market and the fact that this type of vaccine has already shown proof of protection in infants makes this approach probably the most attractive vaccine candidate for a vaccine manufacturer.

The remaining choice to be made is whether to include a *B. parapertussis* component in an improved whooping cough vaccine. Given the fact that inactivated *B. parapertussis* cells work as an injectable vaccine, it stands to reason to assume that the same inactivated cells can be used as an oral vaccine as well. Recent warnings against the rise of *B. parapertussis* all come from countries where the cellular vaccine has been replaced by an acellular vaccine (Watanabe & Nagai, 2004). It is conceivable that using an oral or intranasal inactivated cellular *B. pertussis* vaccine could curb this increasing incidence of *B. parapertussis* induced whooping cough, given the fact that inactivated injectable WCV's provide some protection against *B. parapertussis* in mouse models (David, 2004), and that an attenuated, live intranasal *B. pertussis* vaccine tested in mice also protected against *B. parapertussis* (Mielcarek, 2006). From a development time and risk reduction approach it is probably better to first register an oral and/or intranasal vaccine based on *B. pertussis* only, since this product has been produced and tested before. Directly incorporating an oral or intranasal cellular *B. parapertussis* component without data on the effect of an oral or intranasal inactivated cellular *B. pertussis* vaccine on *B. parapertussis* incidence probably does not warrant the extra investment in terms of time and resources. After sufficient data have been gathered, a *B. parapertussis* formulation could always be included in a later stage if this component is deemed necessary.

The use of an oral or intranasal vaccine is an exciting prospect, both for the vaccinee and the manufacturer. The vaccinee will probably not experience any discomfort after vaccination, while the costs are probably less than those of current acellular vaccines. Rather than focusing clinical trials on protection of infants, it is probably a more strategic choice to first register an oral or intranasal vaccine for adolescents and adults, and only later expand the use to toddlers and infants. This approach reduces costs and time to market, because it is much easier to do a large scale clinical trial in adults than in infants. In the mean time, infants still benefit, since the chances of contracting whooping cough from adults decrease. The use of an oral or intranasal vaccine is also interesting for a manufacturer, since it paves the way to routine adult pertussis vaccination, without the typical decade between an investment and the return on it.

Glossary

ACT	Adenylate Cyclase Toxin, excreted antigen that binds to mammalian cells and induces apoptosis
aCV	low dose booster ACV, containing 30% of the antigen amounts of an ACV
ACV	ACellular Vaccine
BrkA	Bordetella resistance to complement killing, excreted antigen that inhibits the complement pathway that recognizes bacterial cell wall patterns and kills any recognized cells
csACV/WCV	ACV or WCV that contain antigens from circulating strains
DNT	Dermo-Necrotic Toxin, intracellular antigen that causes tissue death
DT	Diphtheria & Tetanus combination vaccine
DTaP	Diphtheria, Tetanus & acellular Pertussis combination vaccine
DTaP-IPV	Diphtheria, Tetanus & acellular Pertussis & Inactivated Polio Vaccine combination vaccine
DTP	Diphtheria, Tetanus & whole cell Pertussis combination vaccine
DTP-IPV	Diphtheria, Tetanus & whole cell Pertussis & Inactivated Polio Vaccine combination vaccine
DTPP	Diphtheria, Tetanus & whole cell Pertussis (whole cell Parapertussis) combination vaccine
FHA	Filamentous HemAgglutinin, outer membrane antigen involved in attachment of *B. pertussis* to the respiratory tract, as well as interfering with the immune system
FIM	FIMbriae, outer membrane antigen involved in attachment of *B. pertussis* to the respiratory tract
GMP	Good Manufacturing Practice, the pharmaceutical industrial standard on how to produce pharmaceutical products
Hypotonic Hyporesponsive Episodes (HHE)	acute reduction in sensory awareness or loss of consciousness, also described as shock or collapse, onset usually within 12 hours after immunisation
LPS	Lipo-Poly-Saccharide, responsible for many of the adverse events in an injectable cellular whooping cough vaccine
PRN	PeRtactiN, outer membrane antigen involved in attachment of *B. pertussis* to the respiratory tract
PT	Pertussis Toxin, excreted antigen with a number of distinct biochemical activities, among which the inhibition of protein synthesis in mammalian cells, leading to apoptosis
QC controls	assays to test a certain aspect of the product such as potency, quantity, purity etc
TCF	Tracheal Colonization Factor, outer membrane antigen involved in attachment of *B. pertussis* to the respiratory tract
TCT	Tracheal Cyto-Toxin, cell wall fragment that kills mammalian cells
waning immunity	the period when protection by a vaccine starts to decrease, for whooping cough vaccines between 3- 7 years
WCV	Whole Cell Vaccine

Intermezzo I. Post vaccination adverse events and public perception: consequences of a successful vaccine

The persistence of the opinion that links pertussis vaccination to neurological damage is understandable given the fact that during the first year of life most congenital neurological conditions in an infant become apparent. Since there are generally at least 3 to 4 vaccinations administered during the first year of life, allegations that an observed neurological condition and vaccination are linked appears plausible. Although a number of investigations into these neurological conditions and vaccination were undertaken, no link could ever be established (Edwards & Dekker, 2004). Still, the consequences of these allegations can be devastating. For example, due to a single controversial publication by Stewart (1977), the vaccination rate in the United Kingdom dropped from 80 to 30%. In Japan, 2 post vaccination deaths, which were later shown not to be vaccine related, led to a decrease in vaccine coverage from 85% to 14% in 1976. The result in both cases was a whooping cough epidemic with 41 infant deaths in Japan, while virtually no deaths occurred before this period (Noble, 1987).

Single scientific publications, biased media attention or unsubstantiated claims from various anti-vaccination organisations usually lead to a drop in vaccination rate, increasing the risk of or leading to an epidemic (Gangarosa, 1998). The whole cell vaccine was not the only vaccine to suffer from this mechanism. A similar situation occurred with regard to a presumed link between measles vaccination and autism where a single allegation by Wakefield (1998) in the UK caused a drop in vaccination rate, even though Wakefield's hypothesis was rejected by a number of authors (review Lewis & Speers, 2003). The sad irony is that parents refusing infant vaccination expose their infant to the considerably higher risk of contracting disease, which can have far more serious consequences than the rare or unsubstantiated adverse effects parents seek to avoid.

Intermezzo II: Limitations of current acellular vaccines

While ACV's have shown less adverse events than WCV's, there are a number of issues related to the use of currently registered ACV's next to the issues mentioned in the main paper that are discussed in more detail in the next paragraphs.

Few B. pertussis antigens were included in clinical trials

Although WCV's cause a number of adverse events, WCV's do contain all virulence factors that allow *B. pertussis* to infect humans. Therefore, the whole cell vaccine elicits an immune response against most, if not all antigens present on the bacterium (Figure 1). In contrast, ACV's contain a maximum of 4 virulence factors, listed in Figure 1 in bold italics. As is apparent from Figure 1, there are at least 5 virulence factors that have never been tested in clinical trials, although these virulence factors were shown to be important for infection. A good example is the BrkA protein, which helps *B. pertussis* to evade killing by the human complement system (Marr, 2007). The complement system can recognize bacterial cell wall or LPS patterns common to most bacteria. A complex chain of protein interactions of the complement system results in puncturing and thereby killing invading bacterial cells. Sera collected from humans that were vaccinated with an acellular vaccine cannot kill wild type *B. pertussis*, while BrkA deletion mutants are killed quite well (Oliver & Fernandez, 2001). This is just one example of an important virulence factor that is not included in any of the ACV's, which could help increase the efficacy of an ACV.

Narrow immunogenic response

Using an ACV instead of a WCV means that the number of antigens against which antibodies are raised went from the entire bacterium down to the 1 to 5 components in an ACV. If an infant vaccinated with a WCV is infected with *B. pertussis*, IgG antibodies will rapidly be produced against all components of the infecting strain that were also present in the vaccine strain. Should there be any components present, which were not present in the vaccine strain, then low levels of IgM antibodies are generated, which are not as effective in assisting in removal of the bacteria as IgG antibodies. When an ACV is used for the primary series of vaccination the situation is markedly different. If an infection takes place, only antibodies are produced against the components that were also present in the ACV. All other surface proteins of the infecting strain elicit low levels of IgM antibodies, which are less effective in assisting removal of the bacteria. Therefore, the variety of antigens in a WCV allows a broader, more robust immune response than ACV's.

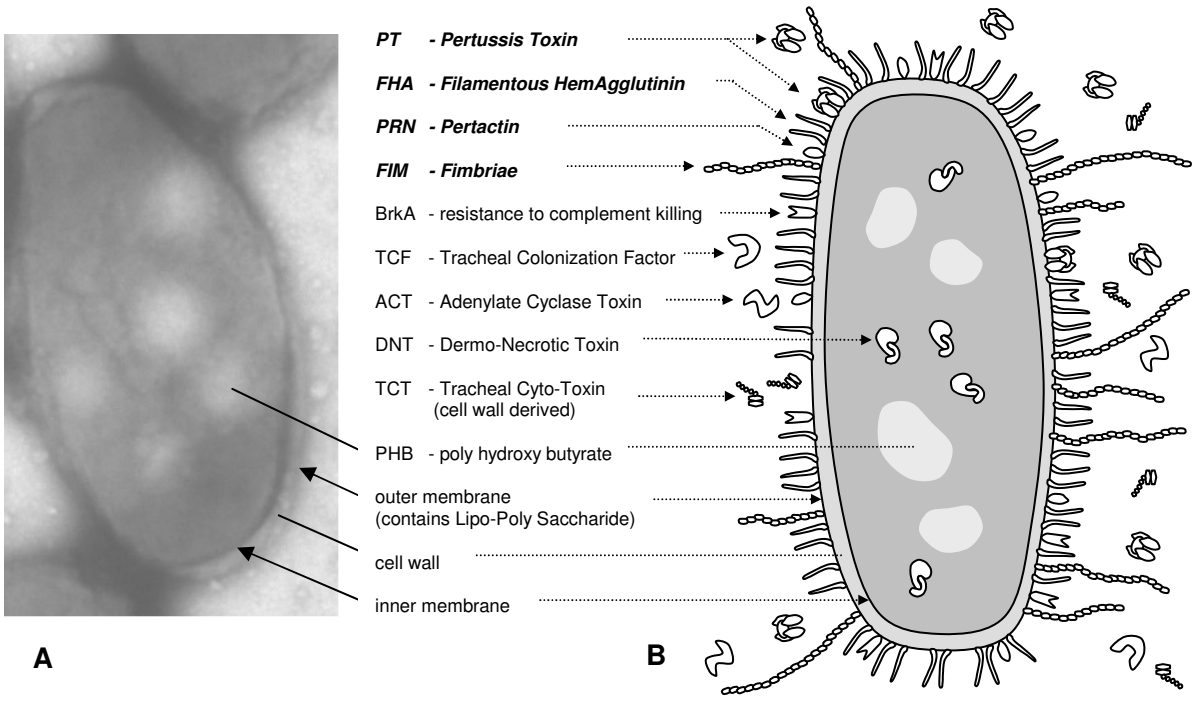

Figure 1. A: Electron microscopic picture of *B. pertussis*. B: schematic representation of *B. pertussis*, including the antigens and structures that play an important role in the ability of *B. pertussis* to cause whooping cough, except for PHB which should be absent from whole cell vaccines. The antigens that are components of acellular whooping cough vaccines are indicated in bold italics.

Antigenic variation

While countries were switching from cellular to acellular vaccines, Mooi (1998) found that the strains that were isolated from patients in the 90's had mutations in PT and PRN compared to the PT and PRN from the vaccine strains. Although the mutations in PT are point mutations, they are located in regions that have been shown to be important epitopes (Mooi, 1998). PRN type 1 is present in all vaccine production strains, yet it is no longer present in strains isolated from patients in the Netherlands. Figure 2 shows that only strains with PRN type 2 and 3 are isolated from patients (Mooi, 2007). He (2003) found that sera from patients that were infected by *B. pertussis* with PRN type 2, hardly cross-reacted with the vaccine PRN type 1, showing that the mutations in PRN are immunologically significant in man. Given the fact that the 3 or more component ACV's all contain PRN, the lack of cross reactivity can potentially undermine the efficacy of the ACV. Although a WCV contains many more antigens, the discrepancy between vaccine and circulating strains may also undermine the efficacy of WCV's. This 'vaccine driven evolution' as postulated by Mooi (2001) may be the reason behind the slow but significant increase in the incidence of pertussis, not just in infants and children, but also in adolescents and adults.

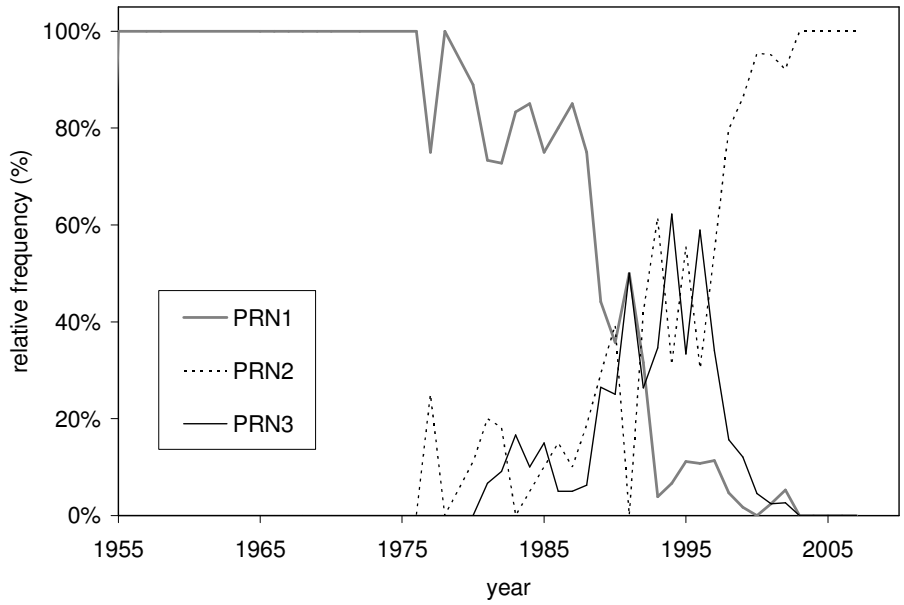

Figure 2. Temporal trends in the relative frequency of PRN variants in the Dutch *B. pertussis* population. The percentage of strains harbouring distinct PRN variants was determined in Dutch strains collected between 1955 and 2007 for each period indicated on the *x*-axis. Strains collected between 1955 and 1975 contained the PRN1 variant only, which is the variant present in the Dutch vaccine strains (from: Mooi, 2007).

B. pertussis infection and WCV's induce different immunity than ACV's

The T-cells involved in immunity after a natural infection in humans are primarily of a Th1 type, i.e. a cellular response mediated by T-cells. WCV's also elicit primarily a Th1 response, while ACV's induce a Th2 humoral response mediated by antibodies (Mascart, 2003). There are some concerns regarding the fact that ACV's elicit a strong Th2 response. Gruber (2001), for example, showed that a Diphtheria and Tetanus (DT) combination vaccine elicited an IgE response against the DT components, while DT vaccine with whole cell *B. pertussis* did not. Mascart (2007) showed the formation of IgE antibodies against a food antigen after ACV vaccination, while this was not observed in infants that had received WCV (Mascart, 2007). A strong Th2 respons at young age has been linked to development of allergies later in life in those groups that are genetically predisposed (Holt, 2003).

Intermezzo III: The trouble with trials

Before a new or modified whooping cough vaccine is tested in humans, the vaccine is tested in animal models to establish proof of concept, and lack of toxic effects. Prior to admitting a vaccine to be registered and sold, all new vaccines are evaluated in a number of clinical trials, which involves administering the vaccine to humans. The most important parameters to investigate in humans are the safety of the vaccine (adverse events), protection against the disease (efficacy), as well as the search for a test that predicts the degree of protection of the vaccine in humans (correlate of protection).

While this approach seems quite straightforward, for each of these 3 parameters a number of issues exists, which makes it difficult to compare one clinical trial with another, and therefore the various products that are tested in different trials. Therefore, unless 2 vaccines are directly compared in the same clinical trial, it is difficult at best to compare the two products. Below are just a few examples that complicate the interpretation of results of clinical trials and the comparison of the various vaccines that were tested in different clinical trials.

Adverse Events: which side effects are observed, and how serious are these?

Although ACV's are generally thought to have a better overall safety profile than cellular vaccines, there are some comments to be made to this widely held belief. After ACV vaccination, a number of adverse effects have emerged that were not seen for the cellular vaccine. The number of extensive local reactions after the 5^{th} dose of DaPT-IPV is as high as 20% or more (Scheifele, 2001). For one particular ACV, hypersensitivity to the alum containing adjuvant resulted in persistent nodule formation in 2-6% of the vaccinees, while approximately 0.5% of the vaccinees had nodules even 4 years after vaccination (Bergfors, 2003). These reactions increase in frequency as the number of vaccinations received increases. Reviewing a large number of ACV clinical trials, Rennels (2003) reported that 2 to 6% of the children that received the 4^{th} or 5^{th} dose of DaPT vaccine showed swelling of the entire limb for several days.

How can these ACV related adverse events be compared with WCV related adverse events in terms of seriousness and vaccine safety? While 1 in 100.000 WCV vaccinees displays convulsions and/or a hypotonic hyporesponsive episode, these always pass with complete recovery within a day. On the other hand, while the persistent nodules and the swollen limbs after ACV vaccination are not directly painful for the vaccinee, the symptoms last longer, and raise questions about their long term effects on the immune system (Mascart, 2003).

Efficacy: how well and how long does the vaccine protect against developing the disease?

All registered whooping cough vaccines provide a high degree of protection against mortality. However, vaccinees can still be infected and develop mild to severe symptoms of the disease and infect others. One of the main problems for clinical trials in the past was the difficulty of isolating *B. pertussis* from suspected pertussis patients, thus unambiguously proving that the observed symptoms were caused by *B. pertussis*. Only the severe symptoms of pertussis such as the persistent cough followed by the characteristic whoop are clearly caused by pertussis. Further confounding factors are the fact that many of the mild symptoms of pertussis can also be caused by many other pathogens or conditions, although serological tests can make the distinction between whooping cough and other types of infections.

During a clinical trial, a number of diagnostic criteria are defined to define what constitutes whooping cough. Usually multiple criteria are used. A very strict case definition is for example 3 weeks of persistent cough, followed by the characteristic whoop, as well as isolation of *B. pertussis* from the patient. A strict case definition means that few vaccinees will be labeled as having clinical pertussis. Therefore, a clinical trial with this case definition will invariably lead to high efficacies for the vaccines tested in the trial. On the other extreme, only a rise in antibody titers and a persistent cough for a week, without further clinical symptoms can also be used as a case definition. In this case, considerably more vaccinees will be classified as cases of whooping cough, and thus the efficacy of the vaccine will be considerably lower than in the first example. Therefore, just like for the adverse events, it is quite difficult to compare vaccines quantitatively if these have not been compared in the same clinical trial.

Correlate of protection: is there a parameter that correlates with protection against the disease in humans?

Currently registered acellular vaccines use 1 to 5 protein virulence factors as vaccine components, without being able to link antibody titers to long term (>2 years) protection against pertussis (Guiliano, 1998, Casey & Pichichero, 2005), even though these vaccines have proven to be efficacious in clinical trials. Despite the thorough analysis of the immunological data in 52 separate trials, to date no serological correlate of protection has been identified for currently registered pertussis vaccines (Casey & Pichichero, 2005). The lack of correlates of protection for ACV's means that there is no method by which to predict with some accuracy whether a vaccine candidate is likely to be successful in humans. The only way to find this out is to do long and expensive clinical trials. Additionally, it is difficult to do clinical trials that show protection against whooping cough in infants,

since most countries vaccinate against whooping cough. This means that the number of cases of whooping cough is not high enough to show statistically significant better protection, unless prohibitively high numbers of infants are included in a clinical trial, i.e. well over 20.000 infants per vaccine composition. As a consequence it is economically quite unattractive for companies to develop and test improved pertussis vaccines. This situation has lead to a *de facto* monopoly of the currently registered vaccines. Fortunately the WHO has recognized this undesirable situation and is working on a number of criteria on how a newly developed vaccine could enter the existing market (Corbel, 2004, Xing, 2007).

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There are two kinds of people, those who finish what they start, and so on.

--Robert Byrne

CHAPTER 7

General Discussion

Whooping cough is, contrary to popular belief, no longer a childhood disease. Although new born unvaccinated infants are most vulnerable for this disease, the economical burden whooping cough presents for a society due to whooping cough infected toddlers, adolescents and adults should not be underestimated. Furthermore, as discussed in **Chapter 6**, whooping cough is not as well under control as for example polio or diphtheria. Rather, the incidence of the disease is rising, which calls for an improved vaccine. Since many of the classical vaccines, including the whooping cough vaccine, are a result of empiric rather than rational research, it is best to re-examine the process choices made in the past and examine if the same choices would be made again with currently available knowledge and technology. The subject of this thesis is the production of *Bordetella pertussis* cells and the antigens it produces, which can serve as the basis for further processing into a vaccine. As such, all steps in this production process from medium to inactivation of the cell suspension are described in this thesis on a fundamental level.

In general, most vaccines that are currently available are composed of proteins and/or polysaccharides that are produced by the pathogen that the vaccine is aimed against. The vaccine against whooping cough is also produced by the organism that causes the disease, *Bordetella pertussis*. Currently available vaccines against whooping cough consist of inactivated whole cells, the Whole Cell Vaccines (WCV), or of purified antigens, the so-called acellular vaccines (ACV). In order to obtain these inactivated cells or purified antigens, the pathogen needs to be cultivated under the conditions that resemble the natural habitat, so that the appropriate antigens are expressed and can serve as a vaccine component.

To be able to improve the growth of *B. pertussis*, its basic metabolism needed to be elucidated further in order to understand which substrates can be used and which substrates cannot, or, even worse, lead to the formation of unwanted by-products. *B. pertussis'* basic metabolism is described in **Chapter 2**. Like in most pathogens, many of the metabolic pathways that would allow growth on a mineral medium and glucose are no longer functional. For example, glucose cannot be utilized by *B. pertussis*, even though glucose residues are present in the cell wall and lipo-polysaccharide (LPS). Initially, *B. pertussis* was cultivated on poorly defined complex media, often supplemented with animal derived substances like horse serum or liver extracts. In the 50's Jebb and Tomlinson undertook two studies that proved to be quite useful for this thesis. Jebb and Tomlinson (1951, 1954) measured which substrates increased the oxygen consumption by *B.*

pertussis in a medium containing yeast extract. Also the medium designed by Stainer (1970) was useful for this thesis. Using a modified version of the Stainer medium as a base, a number of the components that increased the oxygen consumption in Jebb and Tomlinson's studies as well as a number of other components were added to the modified Stainer medium to see if the organism could use these components as substrates. Although *B. pertussis* could not grow on substrates like succinate, malate, fumarate or lactate alone, in combination with glutamate *B. pertussis* could grow faster and to higher cell densities than on glutamate alone. Also, since these compounds contain no nitrogen, modified Stainer medium could be balanced with regard to nitrogen, thus avoiding the accumulation of ammonium in the medium as is the case in all other *B. pertussis* media.

The use of NMR measurements enabled the detection of metabolites consumed and formed, which enabled the composition of a map of *B. pertussis*' metabolic capabilities. An unexpected finding was that the citric acid cycle in *B. pertussis* is not fully functional, the cycle is interrupted between oxaloacetate and iso-citric acid, even though the genes are present and do not contain any clear deletions or other defects (Parkhill, 2003). Another unexpected finding was the formation of globules inside the cells as visible under an electron microscope. On the basis of the excretion of β-hydroxy-butyrate identified with NMR, it was hypothesized that the globules consist of poly-hydroxy-butyrate. Indeed the purified cell extracts did contain PHB as confirmed by NMR. By using lactate and glutamate as main metabolites, PHB formation could be avoided, which resulted in a yield of 8.8 gram biomass per mole carbon consumed. This yield is approximately 20 to 30% higher than what is generally reported (Licari, 1991, Frohlich 1995).

As mentioned earlier, for optimal antigen expression, *B. pertussis* needs to be cultivated under conditions that resemble the natural habitat, i.e. the human respiratory tract. The composition of the cultivation medium therefore needs to be optimized in order achieve high expression of the *B. pertussis* antigens that are known to elicit a protective immune response. This work is described in **Chapter 3**. The expression of Pertussis Toxin (PT) was optimised, as this antigen is representative for all antigens expressed by *B. pertussis*. Several factors are known to influence the expression of *B. pertussis* virulence factors, such as the Na^+ concentration (Frohlich, 1995), the iron concentration (Lacey, 1960) and the temperature. While in the past these parameters were mostly used to examine expression versus no expression, this was the first time that these parameters were tested over a broad range.

Interestingly, the concentration of sodium that was shown to be optimal for growth and cell associated PT expression was 75 mM, rather than the usual value of 150 - 160 mM, also used in physiological saline solutions and other *B. pertussis*

media. This 75 mM is close to the concentration of 69 mM in the fluid of the respiratory tract (Joris, 1993). Also surprising was the impact the sodium concentration had on the optical density of the bacterial suspension. At 25 mM sodium, the optical density was 35% higher than at physiological saline concentration, although the absolute amount of biomass in terms of dry weight was identical. This finding is relevant, since the strength of all cellular whooping cough vaccines, i.e. the amount of biomass per dose, is determined by optical density rather than by a more absolute measurement such as dry weight or total protein. This observation may help to explain the batch to batch potency differences of WCV's, since the amount of sodium in acid hydrolyzed casein, a commonly used medium component, is variable.

Although a low iron concentration positively influenced specific PT production, the iron concentration at which this occurred was too low to support normal growth of *B. pertussis*. The other important virulence factors were not measured, but since these should be expressed in relation to PT, this finding may be useful to develop a whole cell vaccine with a very low number of cells per dose, similar to the whole cell pertussis vaccine produced by the Massachusetts Public Health Laboratories Biologic Laboratories, which only contains 25% of the biomass that is usually present in a WCV. In one of the large clinical trials in Sweden this product was tested and shown to be highly efficacious and to have adverse events comparable to an ACV rather than a WCV (Decker, 1995).

Next to PT expression, also the association of PT to the cells was measured, since PT, and other antigens excreted into the supernatant, are typically lost during the processing of a cellular vaccine. After optimizing the medium for PT expression, the yield of PT per gram biomass reached a level of about 2 mg/g, which was mostly cell associated. Also, the activity of a heat sensitive protease produced by *B. pertussis* was monitored in order to select conditions that favored high expression of antigens, while limiting the degradation of these antigens by the protease. These findings could be relevant for the production of acellular vaccines as well since this protease will be active during processing of any *B. pertussis* suspension. The resulting Thalen-IJssel (THIJS) medium (Thalen, 2006) is suitable for optimal expression of *B. pertussis* antigens, largely associated to the cells, with very limited degradation of the antigens.

As described in **Chapter 4**, the optimized THIJS medium was tested in large scale bioreactors in order to verify the suitability as a medium for WCV production. In terms of dry weight, approximately 1 gram of biomass was produced per liter of medium, which corresponds to about 1200 doses of whole cell vaccine. Next to implementing the THIJS medium, a number of steps of the production process of the conventional cellular vaccine was evaluated in order to minimize batch to batch variability, and to increase the average level of expression of *B. pertussis'*

antigens. There were a number of causes for the batch to batch variation such as the complex media components, a very low inoculum percentage of the production culture, as well as a long storage period prior to heat inactivation of the cell suspension. After dealing with these issues, whole cell vaccine was produced using the THIJS medium, and a modified harvest and inactivation method. Analysis of the vaccine showed that in terms of dry weight, the THIJS vaccine had a 15% lower dry weight content per vaccine dose than the routine vaccine. As observed in **Chapter 3**, this could be due to the fact that the THIJS medium contains less sodium, than the routine B2 medium (75 and 160 mM respectively), which results in a higher optical density reading per gram dry weight for the THIJS suspension. Notwithstanding the lower amount of biomass per vaccine dose, the resulting vaccine had a potency that was 2 to 3 fold higher than the conventional product produced with the same equipment. Furthermore, a two year real time stability study showed that the products resulting from this production process retained the higher potency compared to the conventional product.

While a WCV requires only a small amount of *B. pertussis* cells per vaccine dose, ACV's require approximately 10 to 25 times more *B. pertussis* cells per human dose as starting material from which to purify the antigens present in the ACV. For example, one liter of culture broth of THIJS medium yields approximately 1200 doses of cellular vaccine. Given a fairly high yield of PT of 2 mg/L on THIJS medium, and a frequently used PT content of 25 µg per dose acellular vaccine, this would result in only 80 doses per liter of culture broth. This is a 15-fold lower yield of doses per liter fermentation volume as compared to the yield of a cellular vaccine, without taking the losses during purification into account. Therefore, in order to avoid having to do 10 to 25 times as many batch cultivations, in **Chapter 5** the metabolic circumstances that enable fed-batch cultivation of *B. pertussis* are described. The key parameters to control were the formation of PHB and free fatty acids, which can be done by carefully controlling the feed rate itself and the point at which the feed is initiated. Although the mechanism by which PHB interferes with cell division is not clear, the results in **Chapter 5** indicate that PHB formation is the reason cells stop growing, while free fatty acid formation appears to be a consequence of, rather than the reason for the cells to stop growing. The feed medium and feeding strategy developed in **Chapter 5** lead to a 7-fold higher yield in biomass as compared to a batch culture.

Since the feed medium that was added to the bioreactor contained sodium glutamate, the sodium concentration in the bioreactor will gradually cause the growth rate to decrease, as was shown in **Chapter 3** at higher sodium concentrations. At a certain point, the culture can no longer consume all the substrates that are supplied in the feed, which leads to PHB formation, and ultimately cessation of growth. To increase the cell density further, sodium glutamate would need to be replaced by another substrate since glutamic acid

does not dissolve in high concentrations. In **Chapter 2**, it was shown that α-ketoglutarate can support growth of *B. pertussis* in the presence of ammonium. Added as an acid, α-ketoglutarate can be dissolved in high concentrations and thus serve as a substrate in the feed medium. Although in the batch cultures described in **Chapter 2** α-ketoglutarate leads to the excretion of a number of metabolites and the formation PHB, potentially a carefully controlled feed rate may circumvent this problem and lead to even higher cell densities.

Although the reason for the increasing incidence of whooping cough is still not entirely clear, there are many opinions on how to solve the problem. The challenge of whooping cough is that the situation is complex and requires multidisciplinary input if it is to be controlled better than it is now. However, there are a number of parties involved in order to enable vaccination of infants and toddlers. On the one hand there are the manufacturers that would like their product to be selected instead of their competitors' products. Then there is the government that has to decide about the national vaccination program in which to include whooping cough vaccination preferably with a choice of suppliers, minding public opinion regarding adverse events and at the same time minding the costs of the health care program. A third party is formed by experts from academia and governmental institutes that study whooping cough epidemiology and adverse reactions of whooping cough vaccination. This group is generally the first to notice an epidemic or problems with a vaccine. Even if these various parties only had the interest of protecting infants and toddlers against whooping cough in the best possible manner, it would be difficult at best to reach agreement between these parties given the fact that their backgrounds differ so much. In reality, the situation is even more complex, and the opinions of the experts are often related to the vested interests they represent. Suffice it to say that there are as many opinions on how to deal with whooping cough as there are experts. Therefore this thesis would not have been complete without adding a personal view in **Chapter 6** on how whooping cough could be dealt with from a commercial production and a public health perspective.

In **Chapter 6** the history of cellular vaccines is extrapolated to acellular vaccines, while taking a number of disturbing recent developments with regard to the epidemiology of whooping cough into account. From this analysis it is clear that currently registered vaccines need to be improved so that the improved vaccine (1) enables all age groups to be vaccinated with *B. pertussis* circulating strain antigens, (2) protects against whooping cough induced by *B. parapertussis*, (3) enables earlier protection of infants against whooping cough, (4) causes minimal adverse events after repeated vaccination, and (5) protects longer than currently registered vaccines. A number of scenario's and corresponding vaccine compositions that comply with (part of) these characteristics are examined, using time to market, costs and risks as constraints. The most likely candidates to fulfill

all characteristics are an oral or intranasal vaccine consisting of inactivated whole *B. pertussis* cells, which is based on the fact that an oral vaccine has already shown proof of protection in a phase III study without adverse events, while an intra-nasal vaccine has shown proof of concept in a phase I study. Live attenuated vaccines fulfill many of the 5 characteristics as well, but will most likely take longer to reach the market. At this point it is not clear if a *B. parapertussis* component should also be included in an improved whooping cough vaccine, and what the cost-benefit ratio would be.

B. pertussis was discovered more than a century ago, and 3 generations of vaccines have been developed since then. Notwithstanding the accomplishments in the past, whooping cough vaccine development is still badly needed and an exciting area of research and development. To quote H.H. Cohen, one of the first whooping cough experts in the Netherlands: 'Pertussis, a shrew still to be tamed'.

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SUMMARY

Whooping cough is, contrary to popular belief, no longer a childhood disease. Although new born unvaccinated infants are most vulnerable to this disease, the economical burden whooping cough presents for any society around the globe due to whooping cough infected toddlers, adolescents and adults should not be underestimated. Whooping cough is not as well under control as, for example, polio or diphtheria, even though the vaccination coverage in most of the Western world is quite high. The incidence of whooping cough has been increasing in all age groups for the last 2 decades. The global rise in whooping cough cases did not lessen after the introduction of acellular vaccines a decade ago, or the introduction of booster vaccinations in toddlers. Currently registered vaccines are not well equipped to overcome the rising incidence of whooping cough and therefore, there is a definite need for an improved whooping cough vaccine. Since many of the classical vaccines, including the whooping cough vaccine, were developed in the 50's, it is good to re-examine the process choices made then, and examine if the same choices would be made again with currently available knowledge and technology. The subject of this thesis is the efficient and reproducible production of *Bordetella pertussis* cells and the antigens it produces, which can serve as the basis for further processing into a vaccine. As such, all steps in this production process from medium composition to inactivation conditions of the cell suspension are described in this thesis on a fundamental level.

To be able to optimize growth of *B. pertussis*, its basic metabolism needed to be elucidated further in order to understand which substrates can be used and which substrates cannot, or, even worse, lead to the formation of unwanted by-products. Like in most pathogens, many of the metabolic pathways that would allow growth on a mineral medium and glucose are no longer functional in *B. pertussis*. In order to be able to design a medium that avoids ammonium accumulation, while substrates are metabolized completely, *B. pertussis'* catabolic and anabolic capabilities have to be known. In **Chapter 2** the available metabolic pathways of *B. pertussis* are studied by a number of cultivation experiments. It was discovered that besides the known dysfunctional glycolytic pathway, *B. pertussis'* citric acid cycle is also not fully functional, the cycle is interrupted between oxaloacetate and iso-citric acid. Although ammonium accumulation was avoided by adding carbon sources like α-ketoglutarate, succinate, malate, lactate and pyruvate to a basal medium with glutamate, NMR analysis showed excretion of acetate, aceto-acetate and β-hydroxy-butyrate, thereby reducing the biomass yield in terms of gram biomass produced per C-mole carbon consumed. Aceto-acetate and β-hydroxy-butyrate were also formed in commonly used Verwey, B2 and modified Stainer Scholte medium. Electron microscopy in combination with NMR showed that cells

early on in these cultures contained poly-hydroxy-butyrate (PHB) globules, which disappeared later during the culture, coinciding with the appearance of β-hydroxy-butyrate and/or aceto-acetate. No globules nor metabolite excretion was detected when lactate in combination with glutamate was used as a substrate. Thus, metabolite excretion and ammonium accumulation were avoided, while the yield of 8.8 gram biomass per C-mol consumed compared favorably with literature values, averaging 6.5 gram biomass per C-mol consumed.

In order to be useful as a vaccine component, *B. pertussis* needs to be cultivated under the conditions that resemble the natural habitat, so that all appropriate antigens are expressed and can serve as a vaccine component. However, *B. pertussis* is grown in wide ranges of cultivation conditions and medium compositions, which are known to influence growth rate, antigen production and degradation, as well as the association of the antigen to the cell. In **Chapter 3** the impact of individual parameters known to influence the expression of virulence factors on Pertussis Toxin (PT) production is described. PT production was chosen to represent all relevant *B. pertussis* antigens, since these are all under control of the same locus. Using the medium described in **Chapter 2** as a basal medium, a number of compounds were identified that negatively affect both growth rate and PT production. Also, degradation by proteolytic activity was shown to be an important parameter to monitor, since it significantly lowered the PT yield under certain conditions. Low sodium concentrations, i.e. 50-75 mM rather than the conventional 150-160 mM, significantly increased the growth rate of the organism, the final optical density, as well as the association of PT to the cells. The absolute amount of biomass produced measured as dry weight was similar for all sodium concentrations tested, which is in contrast with earlier literature. While it is known that high iron concentrations inhibit virulence factor production, it was shown that iron-limited growth resulted in very high specific PT production. This finding could be used to develop a whole-cell vaccine with little biomass per dose, reducing whole-cell vaccine toxicity. Finally, the *B. pertussis* strain 509 used in this **Chapter 3** produced 30% more PT at 34 than at 37°C, the latter being a commonly used cultivation temperature. The data in this chapter show that existing production processes for cellular and acellular vaccines can in principle be considerably improved by taking simple measures.

The optimized medium described in **Chapter 3** was used to produce a WCV in standard large scale production equipment in order to assess the characteristics of the resulting whole cell vaccine , which is described in **Chapter 4**. Consistency of production however is a typical problem inherent to cellular vaccines. Optimizing and increasing the consistency of production of whole cell suspensions using product potency as a measure is not possible, since the mandatory animal test to measure potency has little discriminatory power. To circumvent this problem, the work described in **Chapter 4** focused on measuring process

parameters *related* to consistency and potency instead, even though the extent of those relationships could not always be quantified. For example, the antigen content of whole cells can be expected to influence the potency, yet it is quite difficult to establish a relationship between these variables since the limited discriminatory power of the potency test. Critical evaluation and modification of individual process steps lead to 2 optimised production processes, NVP-96 and NVP-THIJS. These processes were compared to the routine production process in terms of antigen and biomass content, potency, toxicity and immunogenicity in mice. The consistency of production for both optimised products was clearly higher than that of the original product for all parameters tested. The biomass content of the NVP-THIJS product was 15% lower than that of the NVP-96 product, while the potency in mice was 2 to 3 fold higher. The stability of the NVP-THIJS product was good over a period of 2 years, while the decline in the potency of both suspensions was comparable.

While a whole cell vaccine requires only a small amount of *B. pertussis* cells per vaccine dose, acellular vaccines require approximately 10 to 25 times more *B. pertussis* cells per human dose as starting material from which the antigens present in the acellular vaccine are purified. Therefore, in order to avoid having to do 10 to 25 times as many batch cultivations, in **Chapter 5** a feed medium and feeding strategy were developed that enable fed-batch cultivation of *B. pertussis* with limited waste metabolism. A feed medium containing lactate and glutamate as substrates was fed to the cultures at various rates. The feed rate and whether or not the feed substrates were completely consumed, significantly influenced cellular metabolism. If lactate was detectable in the culture broth, while glutamate was not, poly-hydroxy-butyrate (PHB) was formed. Any PHB present was metabolized again when glutamate became detectable again in the culture liquid. At higher lactate and glutamate concentrations, free fatty acids were produced. Though toxic, free fatty acids were not the reason cultures stopped growing. The key parameters to control were the formation of PHB and free fatty acids, which can be done by carefully controlling the feed rate itself and the point at which the feed is initiated. Although the mechanism by which PHB interferes with cell division is not clear, the results described in **Chapter 5** indicate that PHB formation is the reason cells stop growing. The feed medium and feeding strategy developed as described in **Chapter 5** lead to a 7-fold higher yield in biomass as compared to a batch culture. However, the PT production stopped at 12 $mg.L^{-1}$,(a 6-fold increase as compared to batch cultures) well before growth stopped, indicating that regulatory mechanisms of PT production may be involved.

Whooping cough is an interesting field to work in due to the multidisciplinary nature of the work involved. Aside from the fundamental and epidemiological science, also commercial manufacturers play a role, as well as governments that have to take public acceptance of vaccines into account, as well as the cost of the

health care budget. Although it is clear that there is a need for an improved whooping cough vaccine, there is no consensus on what such a vaccine should look like. Therefore, in **Chapter 6** the available data on the limitations of currently licensed vaccines are analyzed, thereby defining the characteristics of an improved vaccine. An improved whooping cough vaccine should (1) enable all age groups to be vaccinated with *B. pertussis* circulating strain antigens, (2) protect against whooping cough induced by *B. parapertussis*, (3) enable infants to be vaccinated earlier, (4) cause minimal adverse events after repeated vaccination, and (5) protect longer than currently registered vaccines. A number of scenario's and corresponding vaccine compositions that comply with (part of) these characteristics were examined, using time to market, costs and risks from the perspective of a vaccine developer/manufacturer as constraints. The most likely candidates to fulfill all characteristics are oral or intranasal vaccines consisting of inactivated whole *B. pertussis* cells, given the fact that an oral vaccine has already shown proof of protection in a phase III study without adverse events, and that an intra-nasal vaccine has shown proof of concept in animal studies and safety in a phase I study. Live attenuated vaccines fulfill many of the 5 characteristics as well, but have not been tested in clinical studies yet, thus they will most likely take longer to reach the market. At this point it is not clear if a *B. parapertussis* component should also be included in an improved whooping cough vaccine, and what the cost-benefit ratio would be.

The work described in this thesis can be of use for the production of cellular or acellular whooping cough vaccines. However, the pharmaceutical sector is conservative in its decisions relating to improving or replacing vaccines given the costs involved. Nevertheless, whooping cough incidence is increasing, and it is vital to focus on what the situation will be like in 10 years time, since this is the typical time it takes to develop or significantly modify a vaccine. It is more than a century ago that Bordet discovered *B. pertussis*. Three generations of vaccines have been developed in the meantime, and still there is a need for an improved vaccine, which is why, after having worked more than a decade on this thesis, whooping cough is still an exciting field to work in.

SAMENVATTING

Kinkhoest is, in tegenstelling tot wat vaak gedacht wordt, geen kinderziekte meer. Hoewel zuigelingen het meest gevoelig zijn voor deze ziekte, is de economische schade die kinkhoest veroorzaakt door ziekte van kleuters, adolescenten en volwassenen voor een samenleving waar ook ter wereld aanzienlijk. Kinkhoest is als ziekte niet zo goed bedwongen als bijvoorbeeld difterie of polio, ook al is de vaccinatiegraad in de westerse wereld zeer hoog. De afgelopen 20 jaar komt kinkhoest steeds meer voor, in iedere leeftijdsgroep. De wereldwijde toename van kinkhoest werd niet minder door de introductie van acellulaire vaccins, voor de meeste landen ongeveer 10 jaar geleden. Ook het invoeren van de vaccinatie van kleuters had geen invloed op het totaal aantal gevallen van kinkhoest. De huidige kinkhoestvaccins zijn niet goed toegerust om de wereldwijde toename van kinkhoest te stoppen, zodat er een duidelijke behoefte is aan een verbeterd vaccin. De productieprocessen voor de meeste klassieke vaccins werden ontwikkeld in de vijftiger jaren. Zodoende is het goed om met de huidige stand van kennis en technologie de toen gekozen processtappen opnieuw te evalueren. Het onderwerp van dit proefschrift is de efficiënte en reproduceerbare productie van *B. pertussis* cellen en de antigenen die door *B. pertussis* geproduceerd worden, zodat deze kunnen dienen als uitgangsmateriaal voor de verwerking tot vaccin. Zodoende zijn alle stappen in dit productieproces van medium samenstelling tot en met de inactivatiecondities van de celsuspensie fundamenteel onderzocht en beschreven in dit proefschrift.

Om de kweek van *B. pertussis* te kunnen verbeteren is het noodzakelijk het metabolisme van dit organisme te ontrafelen om te begrijpen welke substraten wel en niet gebruikt kunnen worden, of zelfs tot ongewenste bijproducten kunnen leiden. Zoals de meeste pathogene micro-organismen zijn vele metabole routes van *B. pertussis* die groei op een mineraal medium met glucose mogelijk maken niet meer intact. De ontwikkeling van een medium waarin ammonium niet ophoopt, terwijl alle substraten volledig verbruikt worden is alleen mogelijk als de katabole en anabole metabole routes van *B. pertussis* bekend zijn. In **hoofdstuk 2** zijn de functionele metabole routes van *B. pertussis* in kaart gebracht door het uitvoeren van een aantal kweekexperimenten. Er werd ontdekt dat naast de glycolyse ook de citroenzuurcyclus niet volledig functioneerde. De citroenzuurcyclus is onderbroken tussen oxaloacetaat en citroenzuur. De ophoping van ammonium kon worden vermeden door het toevoegen van koolstofbronnen zoal
α-ketoglutaraat, succinaat, malaat, lactaat en pyruvaat aan een basaal medium met glutamaat. Uit analyses met NMR bleek echter dat acetaat, acetoacetaat en β-hydroxy-butyraat werden uitgescheiden, waardoor de opbrengst van biomassa per C-mol koolstof laag was. Acetoacetaat en β-hydroxy-butyraat werden ook aangetoond in de veelgebruikte Verwey, B2 en Stainer-Scholte media. Electronenmicroscopie in combinatie met NMR metingen toonden aan dat cellen tijdens de eerste fase van de groei poly-hydroxy-butyraat (PHB) deeltjes bevatten.

Deze PHB deeltjes verdwenen later in de kweek, wat samenviel met het verschijnen van β-hydroxy-butyraat en/of acetoacetaat. Wanneer lactaat en glutamaat samen werden gebruikt als substraten werden geen PHB deeltjes of uitgescheiden metabolieten waargenomen. Op deze manier werd ammoniumophoping en uitscheiden van metabolieten voorkomen, bij een opbrengst van 8.8 gram biomassa per geconsumeerde C-mol, wat beter is dan de gemiddelde literatuurwaarde van 6.5 gram biomassa per geconsumeerde C-mol.

Als uitgangsmateriaal voor een vaccin moet *B. pertussis* zoveel mogelijk onder omstandigheden gekweekt worden die overeen komen met het natuurlijke milieu waarin *B. pertussis* voorkomt, zodat alle belangrijke vaccinantigenen zoveel mogelijk tot expressie komen. Echter, in de praktijk wordt *B. pertussis* onder een groot aantal verschillende omstandigheden gekweekt, die invloed kunnen hebben op de groeisnelheid, de productie en afbraak van antigenen, evenals op de mate van associatie van de antigenen met de cel. In **hoofdstuk 3** wordt de invloed van een aantal parameters, waarvan bekend is dat deze invloed hebben op de expressie van antigenen, op de expressie van Pertussis Toxine (PT) beschreven. PT-productie is gekozen als zijnde representatief voor de expressie van alle *B. pertussis* antigenen aangezien deze allemaal door dezelfde locus gereguleerd worden. Uitgaande van het medium dat beschreven is in **hoofdstuk 2**, werd een aantal componenten geïdentificeerd die een negatief effect hadden op zowel de groeisnelheid als de PT productie. Tevens bleek dat afbraak door proteolytische activiteit een belangrijke parameter is om te meten aangezien deze onder bepaalde omstandigheden de PT-opbrengst aanmerkelijk kan verlagen. Een lage natrium concentratie, d.w.z. 50 tot 75 mM in plaats van de gangbare 150 tot 160 mM, had een aanzienlijke positieve invloed op zowel de PT-productie, de groeisnelheid en de associatie van PT aan de cellen. De absolute hoeveelheid biomassa gevormd tijdens de kweek, gemeten als drooggewicht, was voor alle natriumconcentraties hetzelfde, wat in tegenspraak is met eerdere literatuur. Hoewel bekend was dat hoge ijzerconcentraties de productie van antigenen remmen, was niet bekend dat ijzergelimiteerde groei resulteert in zeer hoge specifieke PT-productie. Deze waarneming kan gebruikt worden om een cellulair kinkhoestvaccin te ontwikkelen dat zeer weinig biomassa bevat, waardoor verwacht mag worden dat de toxiciteit omlaag zal gaan. Verder bleek dat de *B. pertussis* stam 509 die gebruikt is in de experimenten beschreven in **hoofdstuk 3**, 30% meer PT te produceren bij 34 ^{o}C dan bij 37 ^{o}C, wat een veelgebruikte kweektemperatuur is voor *B. pertussis*. De data in dit hoofdstuk laten zien dat bestaande productieprocessen voor cellulaire en acellulaire vaccins in principe aanmerkelijk verbeterd kunnen worden door het nemen van eenvoudige maatregelen.

Het medium beschreven in hoofdstuk 3 is gebruikt om cellulair kinkhoestvaccin te maken in bestaande grootschalige productieapparatuur om zodoende het resulterende vaccin te kunnen karakteriseren, hetgeen beschreven is in **hoofdstuk 4**. De reproduceerbaarheid van het cellulaire vaccinproductieproces is

een probleem wat inherent is aan de productie van cellulair kinkhoestvaccin. Het verhogen van de reproduceerbaarheid en het verbeteren van het productieproces is nauwelijks mogelijk door de werkzaamheid van het vaccin te meten, aangezien de daarvoor gebruikte test te weinig discriminerend vermogen heeft. Om dit probleem te omzeilen zijn procesparameters gemeten die *gerelateerd* zijn aan werkzaamheid, al is die relatie niet altijd goed kwantificeerbaar. Het gehalte aan antigenen van de *B. pertussis* cellen bijvoorbeeld, mag verondersteld worden een relatie te hebben met de werkzaamheid, al kan die relatie moeilijk kwantitatief vastgesteld worden. Een kritische evaluatie en modificatie van de individuele processtappen van het bestaande productieproces leverde 2 geoptimaliseerde productieprocessen op, NVP-96 en NVP-THIJS. De hieruit voort komende vaccins werden vergeleken met het routineproces per vaccindosis qua antigeen gehalte, biomassa gehalte, werkzaamheid, toxiciteit en immunogeniciteit in muizen. De reproduceerbaarheid van het productieproces lag voor beide nieuwe processen duidelijk hoger dan het bestaande proces voor alle geteste parameters. Het biomassa gehalte per dosis van het NVP-THIJS product lag 15% lager dan voor het NVP-96 product, hoewel de werkzaamheid in muizen 2 tot 3-voud hoger was. De stabiliteit van het NVP-THIJS product was goed gedurende 2 jaar, terwijl de helling van de afname van de werkzaamheid voor beide producten vergelijkbaar was.

De hoeveelheid B. pertussis biomassa per vaccindosis in een cellulair vaccin is gering. De hoeveelheid biomassa die als uitgangsmateriaal nodig is om de componenten uit te zuiveren voor een dosis acellulair vaccin ligt een factor 10 tot 25 hoger dan de hoeveelheid biomassa die nodig is voor een cellulair vaccin. Om te vermijden dat 10 tot 25 keer zo veel batchkweken uitgevoerd moeten worden, worden in hoofdstuk 5 een feedmedium en feedstrategie beschreven die het mogelijk maken om B. pertussis fed-batch kweken uit te voeren waarbij vrijwel alle substraten doelmatig worden gebruikt. Een feedmedium bestaande uit een geconcentreerde oplossing van lactaat en glutamaat werd toegevoerd naar de bioreactor met verschillende snelheden. De feedsnelheid en het al dan niet volledig verbruiken van de toegevoerde substraten in de bioreactor hadden een grote invloed op het cellulaire metabolisme. Het aantoonbaar aanwezig zijn van lactaat in de bioreactor, in afwezigheid van glutamaat, leidde tot PHB vorming. Als er PHB in de cellen aanwezig was, dan verdween dit zodra glutamaat weer aantoonbaar was in de bioreactor. Bij hogere concentraties van zowel lactaat als glutamaat werden vrije vetzuren gevormd. Hoewel vrije vetzuren toxisch zijn, bleek de vorming van vrije vetzuren niet de reden te zijn waarom de cultures stopten met groeien. De PHB vorming en de vorming van vrije vetzuren zijn de belangrijkste parameters die in de hand gehouden moeten worden. Dit kan bereikt worden door de feedsnelheid en het moment waarop de toevoer gestart wordt nauwkeurig te regelen. Alhoewel het mechanisme waardoor PHB vorming interfereert met de celdeling onbekend is, lijkt het er in hoofdstuk 5 op dat PHB vorming de reden is dat de groei stopt. Het feedmedium en de gekozen feedstrategie zoals beschreven in hoofdstuk 5 leidden tot een 7-voud hogere opbrengst aan biomassa in vergelijking tot een batchkweek. Hierbij moet worden

opgemerkt dat de PT-productie stopte bij 12 mg/L, een 6-voudige verhoging vergeleken met batch kweken, maar ruim voordat de groei stopte. Dit impliceert de PT-productie stopt door B. pertussis' regulatoire mechanismen met betrekking tot de expressie van PT.

Kinkhoest is een interessant veld om in te werken vanwege het multidisciplinaire karakter van het werk. Behalve de fundamentele en epidemiologische wetenschappelijke kanten spelen ook commerciële producenten een rol, evenals overheden die rekening hebben te houden met de publieke opinie enerzijds en de kosten van de gezondheidszorg anderzijds. Hoewel het duidelijk is dat er behoefte is aan een verbeterd kinkhoestvaccin is er geen consensus over waaraan dit vaccin moet voldoen. Zodoende wordt in **hoofdstuk 6** de analyse beschreven van de beperkingen van huidige kinkhoestvaccins, waardoor het mogelijk wordt om de karakteristieken van een verbeterd kinkhoestvaccin te definiëren. Een verbeterd kinkhoestvaccin moet (1) alle leeftijdsgroepen kunnen beschermen tegen de *B. pertussis* stammen die nu geïsoleerd worden, (2) bescherming bieden tegen *B. parapertussis*, (3) eerder toegediend kunnen worden aan zuigelingen, (4) een minimum aan bijwerkingen veroorzaken naar herhaalde vaccinatie, en (5) langer beschermen dan de huidige kinkhoestvaccins. Een aantal scenario's en bijbehorende vaccinsamenstellingen zijn uitgewerkt en afgezet tegen bovengenoemde karakteristieken, waarbij kosten, de tijd voordat een product beschikbaar is en de risico's voor de ontwikkelaar/producent gebruikt worden als criterium om een potentieel te ontwikkelen vaccin mee te beoordelen. De vaccinkandidaten die voldoen aan de meeste karakteristieken zijn een oraal of intranasaal vaccin dat bestaat uit geïnactiveerde cellen, aangezien een oraal vaccin al bewijs van bescherming heeft geleverd in een fase III klinische studie, en het intranasale vaccin bewijs van concept heeft laten zien in diermodellen en veiligheid in een klinische fase I studie in de mens. Levende, verzwakte *B. pertussis* vaccins voldoen aan dezelfde karakteristieken als de orale en intranasale vaccins, maar deze zijn nog niet getest in klinische studies en zodoende zal het langer duren voordat deze vaccins beschikbaar zijn. Op dit moment is het niet mogelijk een beslissing te nemen of een *Bordetella parapertussis* component moet worden toegevoegd aan een verbeterd kinkhoestvaccin, aangezien het onduidelijk is wat de kosten baten analyse is.

De onderzoeksresultaten die beschreven zijn in dit proefschrift kunnen bruikbaar zijn voor de productie van een cellulair of acellulair vaccin. Echter, de farmaceutische wereld is voorzichtig bij het nemen van beslissingen met betrekking tot het verbeteren of vervangen van vaccins gezien de kosten die dit met zich meebrengt. Niettemin neemt kinkhoest toe, en het is belangrijk om na te gaan hoe deze situatie er uit zal zien over 10 jaar, aangezien dit doorgaans de tijd is die nodig is om een vaccin te ontwikkelen of ingrijpend te wijzigen. Het is meer dan een eeuw geleden dat J. Bordet *B. pertussis* ontdekte. In de tussentijd zijn 3 generaties vaccins ontwikkeld, en nog steeds is er behoefte aan een verbeterd vaccin. Daarom is kinkhoest, zelfs na 10 jaar bezig te zijn geweest met dit proefschrift, nog steeds een boeiend onderwerp om aan te werken.

Sometimes you're the statue, and sometimes you're the pigeon.

--Bernie Bickerstaff

DANKWOORD

Het kortste dankwoord ooit zou zijn 'Iedereen bedankt voor alles', of een paar alinea's zonder namen om zeker te weten dat je niemand hebt vergeten. Maar een dankwoord zonder namen is als koffie zonder caffeine, of cola zonder rum: je kunt ’t wel drinken maar ’t heeft weinig effect. Zodoende een wat langere versie, met veel maar lang alle namen van mensen een indruk hebben achtergelaten. De eerste namen laten zich raden. Pap en Mam, jullie hebben me altijd gesteund en gestimuleerd om mezelf verder te ontwikkelen, ook al was het jullie niet altijd duidelijk waar ik nou helemaal me bezig was. De nieuwsgierigheid die Mario en ik als kind al hadden hebben jullie altijd de ruimte gegeven, al ging dat wel eens fout ('Wat gebeurt er als je een rauw ei in heet frituurvet gooit?'). Het hokje waar we met electronica konden prutsen of het in staat stellen om een jaar naar Amerika te gaan na 't VWO zijn daar voorbeelden van. Het pragmatisme, de wil om iets tastbaars te creeren heb ik van geen vreemde, Pap, of dat nu gaat op een manier zoals iedereen het doet of op een manier waarvan iedereen zegt dat het zo niet kan. De belangstelling voor hoe anderen in elkaar zitten ligt waarschijnlijk op m’n X-chromosoom, evenals de wil om lol te maken met anderen, al dan niet in een werkomgeving. Mario en Hiske, ik heb jullie en de kids te weinig gezien de afgelopen jaren. Het onnavolgbare, voor anderen niet grappige en waarschijnlijk alleen door Anneke te volgen gevoel voor humor dat ik met Mario deel hebben we onvoldoende kunnen uitleven de laatste jaren, misschien ook omdat Hiske de servetringen in de vorm van eendjes verstopt heeft? Anneke, eigenlijk wil ik jou ook bij m'n familie noemen: jij bent en blijft de zus die ik nooit gehad heb.

De saaie variant van m'n curriculum valt verderop te lezen, maar bij deze wil ik de mensen bedanken die ik in de loop van de tijd bij het RIVM/NVI heb ontmoet. Als eerste is dat Coen natuurlijk, het levende bewijs dat ontembare werklust en het woord ambtenaar wel degelijk in dezelfde zin kunnen voorkomen. Coen, ik heb erg veel van je geleerd, het ging bij jou altijd om de zaak, nooit om de persoon, al was dat soms pijnlijk voor betrokkenen. Je enthousiasme en werklust waren aanstekelijk. Ook al botste jouw dadendrang en rechtlijnigheid met sommigen binnen het instituut, je slaagde er meestal wel in om dingen gedaan te krijgen. Je liet mij vrij om me te ontwikkelen binnen het China project, en later heb je je er voor ingezet dat ik dit promotieonderzoek kon beginnen binnen het kinkhoestproject, nota bene aangesteld bij SVM door Gijsbert Guijt. Ookal wordt onze relatie niet meer gekarakteriseerd door 'baas B' en 'maatje', dat doet niets af aan m'n waardering voor jou.

Gijsbert bedacht dat ik m'n promotieonderzoek best in 1 dag per week thuis moest kunnen doen, iets waar ik nooit spijt van gehad heb. Natuurlijk vlotte het onderzoek op deze manier langzamer dan bij een normaal promotieonderzoek, voor zover daar sprake van kan zijn, maar dankzij Jan en later Marian zijn er

bergen werk verzet op kweekgebied. Jan, jij bent een van de meest evenwichtige mensen die ik ken, met een sociale intelligentie waar weinigen aan kunnen tippen. Marian, jij laat steeds zien dat je met je eigen normen en waarden denkt, los van dat wat sociaal wenselijk of te doen gebruikelijk is. Kortom, ik leerde al snel dat het goed was om naar je te luisteren, of ik het nu met je eens was of niet. Natuurlijk moesten al die monsters van Jan en Marian ook geanalyseerd worden. Zo zijn een paar duizend monsters door Luc geanalyseerd op PT activiteit, vetzuren, aminozuren en wat dies meer zij. Helaas is Luc na een langdurige, slopende ziekte onlangs overleden. Sommigen definiëren gelukkig zijn als vrede hebben met het heden. Luc was een gelukkig mens, met een geweldige familie. Luc was degene die me in contact bracht met Bert, 'want die kon cystine meten'. Bert bleek niet alleen cystine maar zo'n beetje alles dat een H-atoom bevatte te kunnen meten met behulp van NMR. Een groot deel van het ophelderen van *B. pertussis'* metabolisme was mogelijk dankzij Bert's NMR analyses. Bert, je hebt de zeldzame gave om je te kunnen en willen verdiepen in vele totaal verschillende onderwerpen, en dan ook nog mee te kunnen denken. Natuurlijk mag Janny niet ontbreken, altijd geinteresseerd in 'die vage cursussen' van mij. Al is Janny's AnBi weinig prominent aanwezig in dit proefschrift, deze meetmethode stond wel aan de basis van het karakteriseren van kinkhoestsuspensies en de optimalisatie van de antigeenexpressie op THIJS medium.

Naarmate het onderzoek vorderde ontkwam ik niet aan het feit dat er dierproeven gedaan moesten worden door het toenmalige LCB aan de door LPO gegenereerde kinkhoestmonsters. Niet alleen is de MPT een erg dieronvriendelijke test, de waarden die eruit komen zijn ook nog eens weinig betrouwbaar. Toch waren de eerste uitslagen van het THIJS-product dermate hoog dat er voldoende aanleiding was om het onderzoek op grote schaal voort te zetten. Bij het toenmalige LCB heb ik altijd alle medewerking gekregen van Henk Loggen, Jos Westendorp en Ineke van Straaten in een heel plezierige sfeer. Ineke en Arno waren degenen die 'die THIJS monsters' wel eens wilden testen in hun diermodellen zoals de PSPT en de voorloper(s) van de MTI. Arno, je zegt altijd dat je eigenwijs bent, maar eigenlijk ben je altijd bereid om water bij de wijn te doen als mensen de moeite nemen om echt naar je te luisteren. Don't worry, ik zal het niet verder vertellen. Dankzij jou heb ik me gelukkig bijna niet hoeven te verdiepen in het mijnenveld van de dierproevenstatistiek.

Het opschalen in gebouw U3 naar eerst 350 L en later naar 1000 L was technisch gezien nauwelijks een probleem. Het feit dat deze activiteiten geregeld moesten worden tussen het toenmalige SVM dat de productiefaciliteiten beheerde, en het RIVM dat de producties uitgevoerd wilde hebben lukte uiteindelijk ook. Zo zijn er uiteindelijk toch 6 succesvolle runs op elke schaal gedaan door de Jan, Dennis, Joost, Kyne en Ettore.

Het bespreken van de manuscripten met Coen bij Hans, Kees en later bij Dirk thuis was altijd leerzaam. Hans wil ik vooral bedanken in z'n hoedanigheid als prof. Hans, jij bent een constante factor in mijn biotech loopbaan. Tijdens mijn studie al was ik gefascineerd door je verhaal over de kinetiek van geïmmobiliseerde enzymen en later het succesverhaal van de insectencellen. Je pragmatische manier van redeneren en daar stug mee doorgaan totdat je bij een oplossing uitkomt sprak me toen al aan. Je had het geduld om niet reguliere AIO's te begeleiden zoals Leo, Gerben en ik, een soort lange-termijn investering. Later nam Dirk het stokje van Kees over. Vooral dankzij Dirk kwamen de artikelen daadwerkelijk in de uiteindelijke vorm waarin ze gepubliceerd konden worden. Dirk, van jou leerde ik dat je door een ietwat andere volgorde of structuur een verhaal veel leesbaarder kunt maken, een vaardigheid die ik in 'mijn andere baan' ook goed kan gebruiken. Met name in de laatste fase hebben Hans, Dirk en ook Truus bij nacht en ontij hoofdstukken zitten doornemen en becommentariëren. Truus slaagde er toch altijd in om inhoudelijke inconsistenties te vinden, ondanks een uitpuilende 'voor mee naar huis' tas.

Natuurlijk heb ik die 14 jaar bij het RIVM/NVI wel wat meer gedaan dan materiaal verzamelen voor dit proefschrift, en met veel meer mensen samengewerkt dan hierboven vermeld. Sommigen mensen hebben aan kinkhoest gewerkt, andere aan difterie of tetanus, zoals Gerard van Ginkel, een geestverwant die zich ook altijd af vroeg 'Wat zou dit knopje doen?'. Studenten kwamen en gingen; Stefan den Breeye, Wim van Schijndel, Michel 'ja, dat-wit-ik-wel' van Veldhoven, Dennis Janssen, Martijn Fox en Anita Dekker. Anita kwam steeds terug om weer wat data te verzamelen voor hoofdstuk 5. Zo was er ook nog de DenkTank, een hecht clubje onder leiding van Ben dat uiteindelijk opging in de NVI raad. Een van m'n leukste en tevens leerzame activiteiten was wel het organiseren van de NVI fusie dag, samen met Antoinette. Met name 't stukje waarin een aantal RIVM/NVI prominenten het moesten ontgelden was leuk om te doen. Al was het stukje zelf erg grappig, de zaken die er niet in terecht zijn gekomen waren nog veel leuker! En zo zijn er nog meer zaken op te noemen die alleen door ingewijden te waarderen zijn zoals BPT=Betalen, Proberen, & Terugsturen, dampvoet, het 's avonds testen van experimentele media en de netwerkversie van Doom, Janny en Marion met een onwillige brandslang, een imitatie door Rogier met Ben als lijdend voorwerp, en de omzwervingen van een met regenwater gevulde Jägermeister fles.

Ik heb veel mensen zien komen en gaan op het NVI, totdat ik uiteindelijk zelf ook ben gegaan. Hoewel de overstap naar SynCo een sprong in het diepe was kan ik na 4 jaar concluderen dat ik m'n plek gevonden heb. Mensen zijn mensen, en als je een kamergenoot hebt met een scherp waarnemingsvermogen in combinatie met een onderkoeld gevoel voor humor, collega's waar je meer contact mee hebt dan 'Leuk weekend gehad?', dan zijn op de keper beschouwd de verschillen

tussen 't NVI en SynCo niet zo groot. Naast een verschil in winstoogmerk verschillen eigenlijk vooral de doorlooptijden en daarmee de diepgang van de ontplooide activiteiten. Werd ik bij 't NVI vaak geassocieerd met 'quick and dirty', bij SynCo valt 't woord 'wetenschappelijk' vaak in dezelfde zin als mijn naam. Pierre wil ik bedanken voor het een jaar lang beschikbaar stellen van 1 dag in de week voor het afronden van mijn proefschrift, al is dit tot nu toe de enige timeline die ik niet heb gehaald....

Naast werk is er ook een prive, hoewel zeker op het RIVM/NVI deze zaken vaak door elkaar heen liepen. Zo heb ik Judith en Stef ook werkgerelateerd leren kennen, evenals Pieter en Sanne. Al zien we mekaar niet vaak, ik weet dat je niets hoeft uit te leggen als je mekaar weer ziet. Alletta, Frank en Marja en de rest van 16A, vormen een deel van mij. Dat geldt helemaal voor mensen die ik langer wel dan niet ken; Corike, Angelo en Louise. De studententijd en daarna was vooral door jullie zo ontzettend leuk. Ieder doet nu z'n ding in het buitenland of 'bijna buitenland', en we zien mekaar te weinig, maar ik weet dat we verbonden zijn en blijven.

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CURRICULUM VITAE

Marcel Thalen was born on august 27, 1964 in Heerlen. He graduated from the VWO in 1982, after which he attended the senior year of the Pontiac Township Highschool in the USA from which he graduated with honors. From 1983 to 1989 Marcel studied Molecular Sciences at the Wageningen University, where he received his Master of Science degree in 1989. During his studies he did traineeships at the department of Molecular Biology, studying nitrogen fixation by Rhizobia in soybean. For the Process Engineering group Marcel did a traineeship at Bio Intermediair (now DSM Biologicals) in Groningen, investigating factors affecting the shear sensitivity of hybridoma cells. The last internship was for the Process Engineering at Amgen, Thousand Oaks, in the United States, where he studied operon engineering and optimised fermentations of *E. coli* that produced indigo from glucose.

After his graduation, a brief stay in the (mandatory) military service was followed by working for the Plant Protection Agency in Wageningen, developing detection methods, DNA or protein based, specific for *Clavibacter michiganensis* to detect ring rot in potatoes. From november 1990 until may 2004 Marcel worked in a variety of positions at the Dutch National Institute of Public Health and the Environment (RIVM) in the Sector Vaccines, which later became the Netherlands Vaccine Institute. As part of the China Vaccine Project Marcel was deeply involved in the scale-up of the Dutch vaccine production processes for diphtheria, pertussis and tetanus vaccines from 140 to 1000 L, redefining the production processes in terms of yield, immunogenicity and robustness. After the successful scale up, Marcel lead the development of a robust, potent pertussis whole cell vaccine on an improved chemically defined medium, and was also involved in improving biochemical and animal testing for this product. Most of that work is described in this thesis. The experience gathered was used later as a project leader to remove animal substances from the production processes for diphtheria, pertussis, tetanus, and polio. His last position at the NVI was head of Process Development. Since 2004 Marcel works for SynCo Bio Partners as scientific officer and project manager.

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