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EXPRESSION OF BORDETELLA PERTUSSIS TOXIN SUBUNITS IN BACILLUS SUBTILIS

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SUMMARY

Pertussis toxin subunits (S1-S5) were expressed in *Bacillus subtilis* using a vector with the promoter, Shine-Dalgarno sequence and the sequence coding for the first 7 amino acids of the signal sequence of the α-amylase gene from *B. amyloliquefaciens*. The use of this vector resulted in a high level of intracellular expression of each pertussis toxin subunit as aggregates in the cytoplasm of *B. subtilis*.

INTRODUCTION

Pertussis toxin, composed of five noncovalently linked subunits (S1-S5), is produced by *Bordetella pertussis* at relatively low levels, a maximum of 50 mg PT per liter of culture medium (Suzuki et al., 1983). The purified toxin from the culture supernatant of *B. pertussis* and is used as a component in acellular vaccines against whooping cough.

Due to concern of the safety of vaccines against *B. pertussis*, the use of pertussis vaccine has been discontinued in some countries, with an increase in the occurrence of whooping cough as a result (Kimura, 1986, Wardlaw and Parton, 1983). Therefore, a development of a safer, at least equally efficacious vaccine is an important goal.

The safety of the vaccines could possibly be increased by producing the vaccine components in a nonpathogenic host. We chose to explore *Bacillus subtilis* as a production host, because it is a nontoxic, nonpathogenic gram-positive organism widely used in industry (Thompson, 1988). The gram-positive nature of this organism ensures the lack of endotoxin, a potential problem if *Escherichia coli* is used as the production host for PT components (Burnette et al., 1988). Using

the secretion system based on the promoter and signal sequence of the α-amylase gene from *B. amyloliquefaciens*, we have shown the production of all PT subunits into the culture medium of *B. subtilis* (Runeberg-Nyman et al., 1987, Saris et al. 1990). However, the yields and the stability of all products were not satisfactory. In a preliminary study we have shown that intracellular production of S4 in *B. subtilis* greatly increased its yield (Himanen et al., in press). We now describe an intracellular production system and demonstrate its effectiveness for all PT subunits in *B. subtilis*, thereby providing material for vaccine development studies.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. The coding sequences of the pertussis toxin subunits were isolated as Hind III fragments from plasmids pKTH1779, pKTH1754, pKTH1756 and pKTH1755 (Saris et al., 1990). The *B. subtilis* strains IH6140 (Palva et al., 1983) and IS56 (Smith et al., 1980) were utilized as hosts for the pUB110 (Gryczan et al., 1978) derivatives constructed in this study and for pKTH229 (Himanen et al., in press). LB medium (Miller, 1972) was used for routine bacterial culturing. In the fermentors, a buffered rich medium (Saris et al., 1990) supplemented with varying amounts of glucose (1-5 %) was used. If needed, kanamycin was included at 10 μg/ml final concentration.

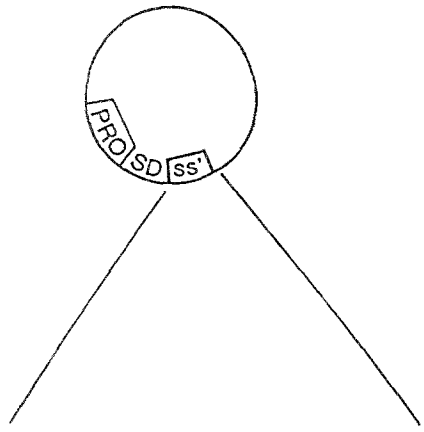
Isolation of DNA, cloning and sequencing. Small-scale preparation of plasmids for screening and testing strains was performed using the method of Holmes and Quigley (1981). General cloning procedures were essentially carried out as described by Maniatis et al. (1982). The nucleotide primers were synthesized on an Applied Biosystems 381A DNA synthesizer using the phosphoramidite method (1981). Nucleotide sequencing was done using plasmid templates essentially according to methods by Sanger et al. (1977) and utilizing the T7 DNA polymerase (Tabor and Richardson, 1987).

Polyacrylamide gel electrophoresis and immunoblotting. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970) with 3.75 % stacking gel and 12.5 % separating gel. The particulate fraction was isolated by centrifugation at 8000 g for 20 minutes. For the immunodetection, the proteins in the gel were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation, MA) by the method of Burnette (1981). The filter was first blocked with 2 % Tween 100, treated with a mixture of typeimmun rabbit sera KH1002, KH1006 and KH1008 (anti-S1, anti-S2,S3 and anti-S4,S5 , respectively), and then with alkaline phosphatase-conjugated second antibody (Promega Corporation, Madison, WI) followed by color development. Several dilutions of the samples and the holotoxin standard were used when the amount of subunit proteins were estimated from the immunoblots.

RESULTS AND DISCUSSION

Construction of expression vectors and hybrid plasmids

The expression vector pKTH39, with the strong α-amylase promoter and the sequence coding for the first seven amino acids of the signal sequence from *B. amyloliquefaciens* has been previously constructed in our laboratory (Palva et al., 1982). We first inserted two EcoRI-HindIII-EcoRI linkers, with the HindIII site in different frames, into the EcoRI site of pKTH39, located



pKTH1781 Met Ile Gln Lys Arg Lys Arg Asn Ser Glu Ala

ATG ATT CAA AAA CGA AAG CGG AAT TCG GAA GCT T

EcoR I Hind III

pKTH1784 Met Ile Gln Lys Arg Lys Arg Asn Leu Ser

ATG ATT CAA AAA CGA AAG CGG AAT TTA AGC TT

Hind III

Fig.1. The vectors for intracellular protein production in *B. subtilis*. Symbols in the plasmid: PRO, the α-amylase promoter of ***B. amyloliquefaciens***; SD, the Shine-Dalgarno sequence of the α-amylase gene; ss', the sequence coding for the 7 first amino acids of the α-amylase signal peptide. PKTH1781 and pKTH1784 were constructed by inserting two different linkers with the HindIII site in different frames into the EcoRI site of pKTH39 (Palva et al., 1982). The linker for the construction of pKTH1784 contained only the AATT part of the EcoRI site (GAATTC) in the 5'-ends, therefore the EcoRI site was not restored in pKTH1784.

downstream of the promoter and the truncated signal sequence, to enable in frame HindIII insertions. This resulted in plasmids pKTH1781 and pKTH1784 (fig. 1). The PT subunit genes, flanked by HindIII sites (Saris et al., 1990), were then cloned into the HindIII site of pKTH1781 or pKTH1784 and screened immunologically for the production of the relevant subunit. Positive clones coding for the S1, S2, S3 and S5 subunits were designated pKTH1785, pKTH1777, pKTH1782 and pKTH1778, respectively. The clones were further verified by restriction enzyme analysis and by sequencing of the joint regions.

Expression of the pertussis toxin subunits in *B. subtilis*

The hybrid plasmids coding for the PT subunits were further transformed into two *B. subtilis* strains, IH6140 (Palva et al., 1983) and IS56 (Smith et al., 1980) with decreased exoprotease activity or with a *relA* genotype, respectively. In fermentor cultivations, the PT subunits were equally well expressed in both strains. The level of expression was very high, up to 1 g per litre of culture, for most of the subunits (table 1). The expression of the S4 subunit, encoded by pKTH229 (Himanen et al., in press), was even higher, up to 3 g/l of culture, which is 6 times more than previously found in conventional batch culture. The expression level of the S1 subunit was lower (100 mg/ l culture) than that of the other subunits. In the fermentor cultures, a considerable part of the S1 subunit was present as degradation products. However, when the bacteria were grown on plates, practically all of the S1 subunit was in full size form, but the expression level was decreased compared to the fermentor cultivation.

When the S1 subunit is expressed from a secretion vector, the majority of the S1 subunit found in the culture medium is truncated (Runeberg-Nyman et al., 1987, Saris et al., 1990), indicating that the S1 subunit is sensitive to degradation by the exoproteases of *B. subtilis*. The S1 subunit also seemed to be sensitive to degradation by intracellular proteases, probably a reason for its relatively low intracellular expression level. Intracellular production of the full size S1 subunit could, however, be achieved in certain growth conditions.

Table 1. Pertussis toxin subunits produced in *Bacillus subtilis* in one-liter scale fermentation.

Subunit	Plasmid	Amount of subunit protein (g/l)[1]
S1	pKTH1785	0.1
S2	pKTH1777	1
S3	pKTH1782	1
S4	pKTH229[2]	3
S5	pKTH1778	1

[1] The amount of subunit proteins were estimated from immuno blots using a mixture of the KH1002, KH1006 and KH1008 sera and pertussis holotoxin as the standard.
[2] Himanen et al., in press .

The S4 subunit is also protease-sensitive when it is secreted from *B. subtilis* cells (Himanen et al., in press). However, the intracellular S4 seems to be very resistant to intracellular proteases, resulting in a very high expression level. It might be that the high hydrophobicity of the S4 subunit promotes the formation of protease resistant aggregates. Another possibility is that the S4 subunit produced intracellularly folds into a protease resistant form. The high expression levels of the PT subunits, produced intracellularly in *B. subtilis*, resulted in aggregate formation in the cytoplasm as shown by their precipitation with the particulate fraction (fig. 2). Interestingly, no inclusion bodies were detected when the producer cells were

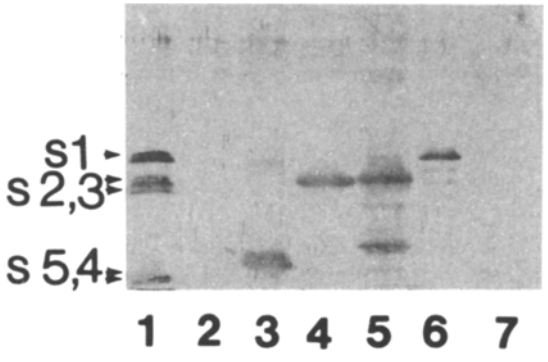


Fig. 2. Western blot analysis of the pertussis toxin subunits produced in *B. subtilis*. A mixture of the rabbit sera KH1002, KH1006 and KH1008 were used to specifically detect the PT subunits. Lane 1: PT purified from *B. pertussis*; lane 2: the cytoplasm, after the removal of the particulate fraction, of the host strain IH6140 (Palva et al., 1983) and the subunit producing strains; lane 3: the particulate fraction of strain IH6140/pKTH1778 expressing S5; lane 4: the particulate fraction of IH6140/pKTH1782 expressing S3; lane 5: the particulate fraction of IH6140/pKTH1777 expressing S2; lane 6: the particulate fraction of IH6140/pKTH1785 expressing S1; lane 7: the particulate fraction of the host strain IH6140.

viewed in a phase contrast microscope, indicating that the PT subunit aggregates formed in the cytoplasm of the *B. subtilis* cells have a looser structure than the PT subunit aggregates formed in *E. coli* cells, which form high density refractile particles (Burnette et al., 1988). The looser aggregate structure might make the renaturation of the subunits as well as the reassembly of the holotoxin easier. This could be of importance as it seems as if the holotoxin is needed (Arciniega et al., 1987, Hausman et al., 1989) in the mouse protection test to achieve high protection.

In conclusion, we have shown that all PT subunits can be produced in high levels in *B. subtilis* showing the potential of *B. subtilis* as a production host for vaccine components.

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