Identification of a *Bacillus subtilis* secretion mutant using a β -galactosidase screening procedure

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High-level synthesis of exportable β -galactosidase (LacZ) fusion proteins in *Bacillus subtilis* results in a lethal phenotype, and has been suggested as a tool for the selection of secretion mutants. We tested a plasmid-based, inducible *lacZ* fusion gene system for this purpose, but frequent mutations *in cis*, which reduced expression of the fusion gene, forced abandonment of the induction-selection strategy. Instead, after modification of the indicator plasmid, a screening procedure for increased basal LacZ activity levels was adopted. This led to the identification of a conditional *B. subtilis* secretion mutant after nitrosoguanidine mutagenesis. At 42 °C, but not at 30 °C, this mutant displayed extreme growth retardation when the LacZ fusion protein was produced, and was also defective in the secretion of subtilisin Carlsberg. The processing kinetics and secretion of a subtilisin Carlsberg–alkaline phosphatase fusion derivative were found to be defective specifically at the non-permissive temperature. The secretion defect was not linked to the *secA/div* locus.

Keywords: *Bacillus*, β -galactosidase, protein secretion, xylose

INTRODUCTION

Genetic approaches, supplemented by *in vitro* methods, have elucidated the protein translocation process in the Gram-negative bacterium *Escherichia coli*. Our present understanding of protein translocation in Gram-positive organisms, e.g. *Bacillus subtilis*, is more limited, despite their industrial utility for the production of secreted proteins, and is largely based on DNA homologies to secretion loci previously identified in other systems, e.g. *secA* (Overhoff *et al.*, 1991; Sadaie *et al.*, 1991), *secY* (Nakamura *et al.*, 1990; Suh *et al.*, 1990), *secE* (Jeong *et al.*, 1993) and *ffh* (Honda *et al.*, 1993).

Only a few genetic approaches have been applied in *B. subtilis* to date. These have revealed apparently novel

secretory loci, e.g. *prsA*, encoding an extracytoplasmic chaperone affecting the amount of active exoenzyme released to the surroundings (Kontinen & Sarvas, 1988; Jacobs *et al.*, 1993), and *sipS*, encoding a non-essential signal peptidase (van Dijk *et al.*, 1992).

To expand these investigations, we chose to adapt the most fruitful genetic approach from *E. coli*, viz. exploitation of the special properties of β -galactosidase (LacZ) fusion proteins which have a functional N-terminal pre-sequence. These 'exportable' proteins display higher enzymic activity when confined to the cytoplasm than when found in the cytoplasmic membrane (Oliver & Beckwith, 1981). Also, synthesis of elevated amounts of such proteins leads to lethality due to abortive transport/'membrane jamming' (Bassford *et al.*, 1979; Phillips & Silhavy, 1990), and consequent disruption of essential exoprotein precursor transport.

Overproduction of LacZ fusion proteins likewise led to lethality in *B. subtilis*, and was suggested as a means for selecting secretion mutants which failed to transport the proteins (Hastrup & Jacobs, 1990; Zagorec & Steinmetz, 1990). As we report here, we were able to isolate a *B. subtilis* mutant with a conditional secretion defect using an inducible expression system for an exportable subtilisin Carlsberg–LacZ (SubC'–'LacZ) indicator protein.

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Abbreviations: APase, alkaline phosphatase; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; NNG, nitrosoguanidine; *O/P_{xyn}*, operator-promoter of xylosidase gene; *phoA*, gene encoding APase PhoA; *subC*, gene encoding subtilisin Carlsberg, SubC; SubCp', SubC36', SubC267', denote respectively only pre-sequence, or prepro-sequence plus 36 or 267 residues of mature SubC; wt, wild-type/parental genotype; *xylR*, xylose repressor-encoding gene; *xynB*, xylosidase-encoding gene.

METHODS

DNA, reagents. Plasmid isolation, DNA manipulation and electrophoresis were carried out by standard methods (Sambrook *et al.*, 1989). DNA fragments were isolated from gels using GeneClean (Bio 101). Enzymes were obtained from New England Biolabs and Boehringer Mannheim; isotope was obtained from Amersham.

Bacterial strains. *B. subtilis* strains 168 *trpC2* (BGSC 1A1), 3G18 *trpC2 purA met* (supplied by J.-I. Flock, Karolinska Institute, Huddinge, Sweden), NIG1121 *his met* and *secA/div-341* derivative NIG1152 (Sadaie & Kada, 1983) were used. Mutagenesis of 3G18 (wild-type: wt) yielded MFJ566. It had no additional auxotrophic requirements. Met⁺ derivatives were obtained by transformation of competent cells (Boylan *et al.*, 1972) with dilutions of *B. subtilis* 168 chromosomal DNA, and selection on minimal medium with relevant auxotrophic requirements.

Media. LB (Sambrook *et al.*, 1989) was used as the rich medium throughout. Chloramphenicol (Cm), erythromycin (Em) or kanamycin (Km) at respectively 6, 5 or 10 µg ml⁻¹, as appropriate, were added to media for plasmid maintenance. Xylose was added to media to induce transcription from *O/P_{xy}*. To detect LacZ activity on plates 40 µg X-Gal ml⁻¹ was used.

Plasmids. pMJ57 (Jacobs, 1995), pMJ67 and pTVB50 (Hastrup & Jacobs, 1990; Jacobs *et al.*, 1993) were derivatives of pSX50 (Hastrup, 1993), a pUB110-based replicon (McKenzie *et al.*, 1987). pMJ550 (this study), pJBA221 and pJBA222 (Jacobs *et al.*, 1993) were based on the pAMβ1 derivative pIL252 (Simon & Chopin, 1988). All supported xylose-inducible synthesis of SubC-related products. pMJ57 encodes SubC; pTVB50 and pMJ550, SubCp'-LacZ; pMJ67 and pJBA222, respectively, SubC36'-LacZ and SubC36'-PhoA; and pJBA221, SubC267'-PhoA, with the SubC moieties defined as in the list of abbreviations. pSX50 derivatives carry a copy of the xylose repressor gene, *xyIR*, and induction ratios were typically 100–150. In pIL252 derivatives, induction ratios were ≤ 10, due to titration of chromosomally-encoded XylR, and low plasmid copy number (Jacobs *et al.*, 1993). pUB110-based plasmids pKTH271, encoding *B. licheniformis* β-lactamase (Simons *et al.*, 1978), and pBS613 (kindly supplied by Georges Rapoport, Institut Pasteur, Paris, France), encoding *B. subtilis* levansucrase, were also employed.

Construction of *subC-lacZ* indicator plasmids. pMJ67 (Fig. 1) has been described (Hastrup & Jacobs, 1990). pTVB50 (Fig. 1) was derived from pMJ67 by replacing sequences between *Hpa*II (just 3' from the *subC*-encoded pre-sequence) and *Hind*III (at the junction with '*lacZ*'), with a synthetic linker (5'-CGTTTCGCGAA-3' and 5'-AGCTTTCGCGAA-3', kindly provided by Novo-Nordisk A/S, Bagsværd, Denmark) having compatible ends bracketing a *Nru*I site. Recloning of *subC-lacZ* to pIL252 (Simon & Chopin, 1988) by ligating pTVB50 474 bp *Eco*RI-*Hind*III *O/P_{xy}-subCp'* and 3.3 kb *Hind*III-*Bam*HI '*lacZ*' fragments with the pIL252 4.7 kb *Eco*RI-*Bam*HI segment yielded pMJ550. Further properties of these plasmids are described in the preceding section.

Mutagenesis. Nitrosoguanidine (NNG) mutagenesis of 3G18 carrying either pTVB50 or pMJ550 was performed in buffer, as described previously (Kontinen & Sarvas, 1988). After exposure, cells were washed twice in buffer, then incubated in LB for 4 h at 30 °C, after which dilutions were plated on LB/X-Gal agar with an appropriate antibiotic, with or without xylose, as described in the text, and incubated at 30 °C. Residual NNG was inactivated with 0.2 M HCl. Batches in which the mutagenesis survival frequency was about 1% were pursued. In the experiment in which MFJ566 was identified, a total of approxi-

mately 20000 colonies was screened, at a density of about 2500 per plate. Blue colonies were scored after 1 d incubation and subsequently disregarded, as these were expected to involve either pre-sequence mutations (Kumamoto & Beckwith, 1983), or the *B. subtilis* chromosomal β-galactosidase genes (Errington & Vogt, 1990; Zagorec & Steinmetz, 1991). After an additional 5 d, approximately 40 newly sectorized or deep blue colonies were picked. Half survived purification, and were tested for possible temperature-related growth defects. Overnight growth without antibiotic selection cured the cells of pMJ550 (Simon & Chopin, 1988).

Mapping mutations to/in pTVB50. A plasmid-borne mutation was indicated by a plating efficiency ratio approaching 1 for 3G18 transformed with plasmid extracted from induction survivors, determined on LB/2% (w/v) xylose, compared with 0% xylose. Mutations were further localized in five pTVB50 variants having unaltered pre-sequences, by individually recloning the 470 bp *Eco*RI-*Hind*III *O/P_{xy}-subCp'* and 1.8 kb *Eco*RI-*Bgl*II *ori* fragments to appropriate, purified fragments of the related pMJ67. Restriction analysis verification of recloning was simple due to the 324 bp larger *subC* fragment size of pMJ67. Recombinant plasmids were tested for transfer of resistance to transformants as described above.

***xyIR* function test.** *xyIR* integrity was tested by monitoring xylose induction of xylosidase encoded by the chromosomal *xyxB* (Hastrup, 1988), and in some cases, also of plasmid-encoded products. In cultures carrying pTVB50 variants, xylosidase activity was measured after growth at 30 °C to OD₆₀₀ 1.5 in LB with 0.2% (w/v) xylose. SubC'-LacZ activity of these cultures was measured after growth to OD₆₀₀ 0.5 in LB, and a further 1 h incubation with 0.2% xylose. Xylosidase activity was determined for 3G18 and MFJ566 cultures, sampled regularly between OD₄₅₀ 0.5 and 8.0, during growth at 30 °C, and at 42 °C, in LB containing 0.05% (w/v) or 0.2% xylose. Xylosidase activity versus OD₄₅₀ for both strains was the same under all conditions tested.

Plate screening of LacZ, temperature-sensitive phenotypes. LacZ phenotype was determined on LB/X-Gal agar after 28–40 h incubation at 30 °C, unless otherwise stated. Mutants were tested for temperature-sensitive growth by quadruplicate streaking to single colonies on LB agar with and without xylose, followed by incubations at 30 °C and 42 °C.

Enzyme assays. The activities of xylosidase (Hastrup & Jacobs, 1990), alkaline phosphatase (APase) (Jacobs *et al.*, 1993), exoprotease and LacZ (Jacobs, 1995), α-amylase and β-lactamase (Kontinen & Sarvas, 1988) were assayed as previously described.

Disc sensitivity assay. LB cultures of Met⁺ derivatives of 3G18 and MFJ566, and their non-induced pMJ550 transformants, growing exponentially at 30 °C, were diluted to identical cell densities and spread on LB agar. Discs (5 mm) with respectively 6, 15, 5, 30 and 5 µg of Cm, Em, Km, novobiocin and rifampicin were tested. Similarly, discs with 10 ml of 1% solutions of the detergents sodium dodecyl sulphate, Sarkosyl and Triton-X 100, and 0.1 M CoCl₂, Na₂Cr₂O₇ and CuSO₄, were tested. Clear zones of growth inhibition were measured after 18 h incubation at 30 °C.

Immunoblot analysis and cell fractionation. Immunoblotting of cell and medium samples was as described for PhoA (Jacobs *et al.*, 1993) and SubC (Jacobs, 1995). Membranes were prepared from 10 ml culture by lysozyme treatment of cells (0.5 mg ml⁻¹, 37 °C, 15 min) in the presence of 1 µg DNase I ml⁻¹ and 10 mM MgCl₂, and sonication to complete lysis (Branson sonifier 250, 30 bursts at output and duty cycle of 4:5). A clearing

Fig. 1. Maps of *subC'*-*lacZ* indicator plasmids. pMJ67 (8.1 kb) has been described (Hastrup & Jacobs, 1990); pTVB50 (7.8 kb) and pMJ550 (8.1 kb) were successively derived from it (see Methods). The diagrams are not to scale, and restriction sites indicated are not necessarily unique. *O/P_{xyn}*, *B. subtilis xynB* operator-promoter; *subC'*-*lacZ*, *SubC'*-*LacZ* fusion gene (diagonal stripes denote *subC* pre-region; vertical stripes, pro- and 36 codons of mature region; solid bar, '*lacZ* moiety'); *xylR*, xylose repressor gene (horizontal stripes); *cat*, Cm transacetylase gene; *erm*, Em resistance gene (open boxes); *ori110*, *ori*pAMβ1, replication regions derived from, respectively, pUB110 and pAMβ1. The direction of *ori* in pMJ67 was previously indicated erroneously (Hastrup & Jacobs, 1990).

centrifugation step (10 min, 1000 g) was followed by membrane collection (200 000 g, 45 min). The gelatinous membrane pellet was resuspended in water and proteins were precipitated, and further treated, as before (Jacobs *et al.*, 1993). Coomassie staining of samples in protein gels was used to normalize immunoblot sample loadings for variable membrane yields.

Pulse-chase labelling and SubC-PhoA immunoprecipitation. These were performed as previously described (Jacobs *et al.*, 1993).

DNA sequencing. Sequencing of drop-dialysed (Marusyk & Sargeant, 1980) double-stranded plasmid DNA was performed with a Sequenase kit (USB), and universal *lacZ* primers.

Sporulation frequency. Dilutions of 10 ml cultures grown at 30 °C for 24 h in Sporulation medium (Schaeffer *et al.*, 1965) were titrated on LB agar before (c.f.u.) and after (spores) heat-treatment (80 °C, 15 min). Sporulation frequency was calculated as (percentage spores)/(c.f.u.).

RESULTS

lacZ fusion expression is detrimental to *B. subtilis*

SubC-*LacZ* fusion protein overproduction from plasmid pMJ67 has a lethal effect in *B. subtilis* (Hastrup & Jacobs, 1990). Studies in *E. coli* have shown that mutations which reduce translocation of an exportable *LacZ* fusion protein can result in its cytoplasmic accumulation (and a *Lac*⁺ phenotype), and may also reduce the deleterious effects of membrane jamming (Schatz & Beckwith, 1990). pTVB50, identical to pMJ67 but with a smaller *subC* moiety (Fig. 1), was tested as a tool for the identification of translocation mutants. In pMJ67 and pTVB50, *subC'*-*lacZ* expression is under xylose control via the xylosidase operator-promoter, *O/P_{xyn}*. We reasoned that employing the relatively simple xylose system, rather than the native *subC* promoter, which is regulated by many pleiotropic factors (Jacobs, 1995), would reduce the chance of isolating mutants defective in *SubC'*-*LacZ* synthesis, rather than its translocation. *O/P_{xyn}* also permitted *subC'*-*lacZ* expression manipulation by alterations in xylose concentrations. Increasing xylose levels caused progressively higher *LacZ* activity in 3G18(pTVB50),

concomitant with colony size and plating efficiency reductions. These were associated with gross cell elongation and lysis. There was an abrupt fall in plating efficiency from 0.03–0.83 to 3.3–8 × 10⁻⁵, as inducer was increased from 0.2 to 2% (w/v). Colonies surviving on 2% xylose were strikingly heterogeneous in size and *LacZ* phenotype, suggesting that a mutational event was required to counteract the effects of *SubCp'*-*LacZ* overproduction. These observations confirmed data obtained with pMJ67 (Hastrup & Jacobs, 1990).

LacZ⁺ survivors have plasmid mutations mainly in *xylR*

Many *E. coli* secretion factors are essential for cell viability (Schatz & Beckwith, 1990); the ratio of conditional to non-lethal secretion locus mutants obtained can be as high as 1:5 (Kumamoto & Beckwith, 1983). We thus sought conditional *B. subtilis* secretion mutants among *LacZ*⁺ 3G18(pTVB50) survivors of *SubCp'*-*LacZ* induction on 2% xylose agar. However, no clones displaying temperature-sensitive growth were found among several hundred such spontaneous or chemically-mutagenized survivors. In all cases examined, the xylose resistance could be transferred to competent cells by plasmid extracted from the mutant. We expected these *cis* mutations to be mainly in the *subC* pre-sequence region (Kumamoto & Beckwith, 1983), but sequencing showed that this was not the case in 20 randomly chosen mutants. Recloning experiments and/or *xyIR* function tests on 11 mutant plasmids localized the resistant phenotype to *O/P_{xyn}* in a single case. In all others, the lesion was in *xyIR*. Hence survival was due to *SubCp'*-*LacZ* synthesis reductions to non-lethal levels by changes primarily in the repressor protein, or at the operator/promoter.

Modifications to the *subCp'*-*lacZ* system

To reduce the rate of indicator plasmid mutations, *subCp'*-*lacZ* was recloned to a different replicon, yielding pMJ550, which does not carry *xyIR* (Fig. 1). 'Theta'-

Fig. 2. Effect of temperature shift on SubC secretion. Fresh overnight cultures of wt 3G18 Met⁺ (pMJ57) and mutant MFJ566 Met⁺ (pMJ57) were diluted to OD_{450} 0.05 in 200 ml LB containing Cm and 0.05% xylose, and grown with moderate agitation in 1 l baffled flasks at 30 $^{\circ}\text{C}$ to OD_{450} 0.25. Cultures were divided into two aliquots, transferred to prewarmed 500 ml baffled flasks, and incubated for a further 3 h either at 30 $^{\circ}\text{C}$ or at 42 $^{\circ}\text{C}$, during which time samples were withdrawn for exoprotease activity assays and immunoblotting. As no SubC-specific signal was obtained in blots from any cell fractions at 30 $^{\circ}\text{C}$, nor from the 42 $^{\circ}\text{C}$ mutant membranes, the experiment was repeated with 0.2% xylose, and cell and membrane samples prepared. (a) Exoprotease activity. Exoprotease activities were normalized for cell density at the indicated sampling times. Final sampling occurred at times corresponding to approximately 1 h (30 $^{\circ}\text{C}$), and 2 h (42 $^{\circ}\text{C}$) after the cessation of exponential growth. Background exoprotease activity (i.e. not due to SubC) was not detectable under these conditions of early xylose induction. Open symbols, wt; filled symbols, mutant; circles, 30 $^{\circ}\text{C}$; squares, 42 $^{\circ}\text{C}$. (b) Subcellular distribution of SubC. Samples were taken at 3 h post-shift, and immunoblotted. In this experiment all the SubC proteins migrated as doublets due to incomplete denaturation: reboiling with additional β -mercaptoethanol caused a shift of most (but not all) material from the upper band of each doublet to the lower, i.e. to the position at which they normally run. Growth temperature, xylose concentration, cellular fraction (S, medium; C, cell; M, membrane) and culture volume equivalent are indicated. The positions of size standards and SubC species are shown. The 38 kDa proSubC was never observed in 42 $^{\circ}\text{C}$ samples (Jacobs, 1995). Lanes 1, 3, 5, 7, 10, wt; lanes 2, 4, 6, 8, 11, mutant; lane 9, control 3G18 Met⁺ (no subC plasmid).

replicating plasmids (like the pAM β 1-based pMJ550) have greater structural integrity (Jannire *et al.*, 1990) than plasmids (like pMJ67 or pTVB50) which multiply by a rolling-circle mechanism. This factor, the low copy number of pMJ550, and the reduction in *xy/R* dosage to the single chromosomal copy (whose integrity could readily be tested), were expected to counteract the

tendency of *O/P_{xy}*, and especially *xy/R*, to mutate. As a result of the modifications, the induction ratio of 3G18(pMJ550) cultures differed markedly from 3G18(pTVB50): basal/maximal activities were respectively 88/150 units and 2.3/1300 units. Maximal activity from the new plasmid was achieved at lower xylose levels (0.005 vs 0.2%–2%). In sharp contrast to

Fig. 3. Synthesis and processing of SubC36'-PhoA. Cultures of wt 3G18 Met⁺ (pJBA222) and mutant MFJ566 Met⁺ (pJBA222) were grown at 30 °C in modified Spizizen's medium containing Em to OD₄₅₀ 0.25, then half of each culture was shifted to 42 °C, as described in the legend to Fig. 2. At OD₄₅₀ 1, half of each culture was induced by addition of xylose to 0.2%. Pulse-chase experiments were conducted on aliquots of induced cultures 2 h later. Samples were withdrawn for APase activity determination at hourly intervals up to 5 h post-shift, and for immunoblotting at 3 h post-induction. The results are shown as an autoradiogram of pulse-labelled SubC36'-PhoA. The positions of size markers, SubC'-PhoA pre-pro- and pro-form precursors, as well as fully processed mature SubC'-PhoA are shown. EP denotes the position of authentic *E. coli* PhoA. (a), (b) Unfractionated cultures grown at 30 °C (a) and at 42 °C (b); (c) cultures grown at 42 °C and separated into cell (C) and medium (S) fractions.

3G18(pTVB50), 2% xylose did not detectably reduce 3G18(pMJ550) colony size.

Isolation of secretion mutant

Variations in LacZ fusion protein enzymic activity with their subcellular location in *E. coli* were most pronounced at low synthesis levels (Oliver & Beckwith, 1981). We thus screened colonies of 3G18(pMJ550) for raised non-induced LacZ activity on LB/X-Gal agar after NNG mutagenesis.

Among 20 such purified mutants, one displayed severe temperature-dependent growth inhibition, but only when *subCp'*-lacZ was induced: colonies grown at 42 °C on 0.2% xylose agar averaged 0.5 mm in diameter versus 6 mm for the wt. Under other conditions the size of this mutant was only slightly reduced relative to the wt. The cured mutant, called MFJ566, had the same auxotrophic requirements as the wt. However, satisfactory growth in minimal media of mutant carrying either pMJ550 or pBS613 could not be achieved, and MFJ566 was sensitive to 0.1 mM cobalt salts when carrying plasmids encoding SubC-PhoA fusion proteins. These problems were not encountered with a variety of other test plasmids, which also encoded secretory proteins. Sensitivity assays revealed that membrane permeability, and tolerance towards a wide range of agents, was not detectably altered in MFJ566. MFJ566 was examined for possible secretory defects as described below; the secretory status of the other 19 mutants was not examined.

Elevated SubCp-LacZ activity in MFJ566

Confirming the results of the screening method, basal, as well as induced LacZ activity in exponentially growing MFJ566(pMJ550) were slightly elevated at 30 °C over parental levels, e.g. 15 vs 10 Miller units at OD₄₅₀ 0.25, with 0.005% xylose. As chromosomal *xylR* function testing showed that this elevation was not due to defective xylose regulation, the most likely explanation was that a reduced fraction of synthesized SubCp-LacZ was transported from the cytoplasm.

For technical reasons we could not demonstrate reduced translocation directly with SubCp-LacZ. The SubCp-LacZ fusion protein displayed no enzymic activity at 42 °C, precluding assay for assessment of cytoplasmic accumulation. Pulse-chase labelling could not be used to monitor the kinetics of translocation-associated pre-peptide removal (Phillips & Silhavy, 1990), because MFJ566 carrying SubC-LacZ-encoding plasmids failed to grow satisfactorily in a variety of minimal media.

As an alternative, we examined the effect of growth at 42 °C on the secretion of proteins that were related to SubCp'-LacZ.

SubC secretion defective at 42 °C

Mutant cultures in which synthesis of SubC had been induced prior to a temperature-shift from 30 °C to 42 °C

had greatly reduced exoproteolytic activity relative to the wt (Fig. 2a). This effect was highly reproducible: in three experiments, there were no significant differences at 30 °C between the low activities seen in wt and mutant. At 42 °C, however, while exoproteolytic activity accumulated rapidly in the wt, there was no net increase in the mutant. These measurements correlated with amounts of extracellular SubC protein detected in immunoblots (Fig. 2b). But as cell- or membrane-associated SubC precursors were also significantly reduced in MFJ566, it was essential to determine whether this was due to differential synthesis, or to post-translational events. To resolve this question we performed kinetic studies employing SubC'-PhoA hybrid proteins (Jacobs *et al.*, 1993).

SubC'-PhoA processing kinetics at 42 °C

Temperature-shift experiments (Fig. 3) were performed on wt and mutant cultures after induction of the pJBA222-borne *subC36'*-*phoA* gene (Jacobs *et al.*, 1993). There were no differences between mutant and wt in either measured APase activities or amounts of anti-PhoA specific protein in culture fractions at 30 °C (data not shown). But at 42 °C both SubC36'-PhoA in the medium fraction as well as APase activity were reduced several-fold in the mutant (data not shown), reflecting the results with SubC. Crucially, pulse-chase analysis demonstrated similar rates of synthesis of SubC36'-PhoA in wt and mutant: there were no significant differences in the amount of incorporated isotope at the end of the pulse period (Fig. 3a-c; 0, 1 min). It was notable that, specifically at 42 °C, cell-associated precursor forms persisted longer in the mutant, and that a smaller fraction of labelled protein was chased into the medium fraction ('S'), compared with the wt, consistent with our steady-state observations. Similar steady-state and kinetic data were obtained with pJBA221-encoded SubC267'-PhoA (data not shown).

Effect on secretion of other enzymes

To determine whether the defect in MFJ566 affected exoenzyme secretion in general, we examined the effect of a temperature-shift on a number of other enzymes. Activity levels of chromosomally encoded AmyE (α -amylase) in cured MFJ566 at 6 h post-shift were depressed to 60% of wt levels at 42 °C, while they were 85% relative to wt at 28 °C. In contrast, no effect on the secretion of a heterologous β -lactamase was found when cultures carrying pKTH271 were studied. The colonial morphology of MFJ566(pBS613) induced to overproduce levansucrase on 2% sucrose agar was strikingly different from the wt control at 42 °C; colonies were much less mucoid, suggesting less sucrose-derived levan around the colony. We could not confirm any effect on processing of levansucrase as MFJ566(pBS613) did not grow adequately in minimal medium.

Mutation in MFJ566 is not linked to *secA*

E. coli secA (Oliver & Beckwith, 1981; Kumamoto & Beckwith, 1983), *secB* (Kumamoto & Beckwith, 1983) and

secD (Gardel *et al.*, 1987) loci were identified by screening regimens similar to that applied here. As the *B. subtilis secA* homologue is known, we could readily test whether the defect in MFJ566 involved *secA* by exploiting the viability of MFJ566(pMJ550) at 42 °C (in the absence of xylose). At this temperature the *div-341 secA* mutant *B. subtilis* NIG1152 cannot grow (Sadaie & Kada, 1983). If the defect in MFJ566 was *secA/div* associated, transformants of NIG1152(pMJ550) with MFJ566 DNA, selected at 42 °C, would be inhibited on 0.2% xylose agar. As none of 93 such transformants displayed xylose inhibition, we concluded that the mutation in MFJ566 did not reside in the *secA/div* gene. NIG1152(pMJ550) itself was not growth-inhibited on xylose plates at its semi-permissive temperature of 37 °C.

DISCUSSION

We have described a lethal phenotype resulting from induced overproduction of SubC'-LacZ fusion proteins in *B. subtilis*. As the level of inducer was raised from 0.2 to 2% xylose, corresponding to only a 2-3-fold increase in amount of xylose-regulated product (unpublished observations), there was a striking drop in viability. This probably defines the titration point of a component required for protein secretion.

Exploitation of this lethal phenotype for the selection of *B. subtilis* secretion mutants was impeded by a high mutation frequency in the xylose expression plasmids employed (Hastrup, 1993). Under the selective pressure, *xylR* mutated frequently to a dominant 'super-repressor' phenotype, and mutant plasmid was rapidly established as the sole species. We did not examine whether the mutant repressor proteins were altered in interaction with the operator/promoter, or with the inducer molecule. In other studies, we have noticed that *xylR* variants encoding a less effective repressor arise frequently during construction of pSX50 derivatives, with no apparent advantage to the host. It is likely that alternative systems that have been proposed for isolating *Bacillus* secretion mutants (Zagorec & Steinmetz, 1990; Nagarajan & Borchert, 1991) will be hampered by similar problems related to the indicator system regulation. These difficulties may be exacerbated by employing plasmids which replicate via a single-stranded intermediate, and are therefore inherently highly mutable (Gruss & Ehrlich, 1989).

A screening procedure demanding a smaller proportion of the poison LacZ hybrid protein to be excluded from the cytoplasmic membrane than did our original selection led to identification of the mutant MFJ566. By the rationale behind the screening method, the elevated LacZ activity in MFJ566 at 30 °C would be due to a reduced ability to transport the LacZ fusion protein from the cytoplasm. This mutant displayed a temperature-dependent, heightened susceptibility to the deleterious effects of *subC-lacZ* induction.

Consistent with a conditional secretory defect, temperature-shift experiments with SubC and SubC'-

'PhoA hybrids, as well as α -amylase, revealed little or no effect on the level of secreted protein at 30 °C, but a significant deficiency after a shift to the non-permissive 42 °C.

Kinetic analysis of SubC⁻'PhoA demonstrated that this defect correlated with conversion of a much smaller fraction of labelled precursor forms to extracellular protein, compared to the wt (Fig. 3b, c). Increased exoproteolytic degradation, specifically in the mutant, might explain such observations, but we do not believe that this applies to MFJ566. There was evidence of extracellular proteolysis, leading to reductions in the amount of mature PhoA at late chase times, apparent in both wt and mutant, at 30 °C as well as 42 °C (Fig. 3a, b, c). However, the intensity of degradation products smaller than PhoA suggests that post-translocational proteolysis could not be the sole factor accounting for the lower steady-state levels of extracellular enzymes in the mutant at 42 °C. In addition, the steady-state levels of SubC (Fig. 2) would argue strongly in favour of diminished production also of other, endogenous, exoproteases in the mutant. Apart from exoprotease levels, incorrect exoprotein folding could lead to higher rates of degradation. In the well-characterized *B. subtilis* *prsA* mutants, exoprotein folding is believed to be deficient, and extracellular proteolytic breakdown results in low exoenzyme levels. In these mutants, the kinetics of pre-sequence removal are notably unaffected (Jacobs *et al.*, 1993). In contrast, in MFJ566(pJBA222) this process is slower at 42 °C (Fig. 3b, c), suggesting that entry into the secretory pathway is less efficient. For these reasons it seems likely that the lower extracellular levels of PhoA, and other exoproteins, are a result of reduced translocation, rather than post-translocational degradation.

For comparative purposes, we performed identical experiments on pJBA222-carrying derivatives of the *B. subtilis* *secA* mutant and its wt control (but shifted up to 37 °C instead of 42 °C). The resulting activities and kinetics were almost indistinguishable from those for MFJ566(pJBA222) (data not shown), and specifically showed, at the non-permissive temperature, a similar degree of precursor persistence in the mutant, and the same striking differences between wt and mutant in amounts of cell-associated mature PhoA and extracellular PhoA at, respectively, early and late chase times (Fig. 3c). We therefore attribute the fading cellular SubC⁻'PhoA signal in MFJ566 and *div-341* to intracellular proteolysis, due to a failure to engage with the secretory machinery. Taken together, the kinetic data, and the lower membrane-associated steady-state levels of SubC (Fig. 2b), suggest that proteolytic precursor breakdown in MFJ566 occurred at an early stage, prior to membrane association. Indeed, since *secA* in MFJ566 was intact, breakdown probably occurs prior to association with SecA: in *E. coli*, pre-secretory proteins complex first with SecA, and are then passed to the integral membrane components of the secretion apparatus.

We were unable to further characterize the genetic nature of the defect in MFJ566. The parental 3G18 had been

chosen for mutagenesis as its colonial morphology facilitated the estimation of LacZ activity on plates. 3G18 proved resistant to PBS1 transduction. Mapping was additionally hampered by exceedingly low conjugation frequencies, revealed in model transformations with two unlinked chromosomal markers, compared with derivatives of the canonical strain 168. We thus cannot conclude at present whether a single chromosomal alteration accounts for the whole phenotype of MFJ566, nor whether the diminished secretory proficiency in MFJ566 is due to a lesion in a component of the secretory machinery, or to a less direct effect. This would require the generation of additional alleles in a different genetic background.

While not strictly components of the secretion machinery, *E. coli* heat-shock proteins DnaJ, DnaK, GroEL and GroES (Kusukawa *et al.*, 1989; Phillips & Silhavy, 1990; Wild *et al.*, 1992) facilitate protein translocation in their capacity as molecular chaperones, maintaining pre-secretory proteins in a translocation-competent conformation. Chaperone SecB additionally targets some proteins to the secretory apparatus (Hartl *et al.*, 1990). The heat-shock response, or overproduction of DnaK and DnaJ, can suppress export defects in *secB* mutants (Altman *et al.*, 1991; Wild *et al.*, 1992). *B. subtilis* homologues of the above-mentioned heat-shock proteins have been identified (Zuber & Schumann, 1994). Acting at a very early stage of translocation, chaperones display a marked selectivity in their interactions (e.g. Kumamoto & Francetic, 1993). These features make it likely that the defect in MFJ566 involves a chaperone: the variable magnitude of effects on secretory proficiency, and viability, of MFJ566 derivatives secreting different exoproteins suggests a variable requirement for the defective factor. The amount, and nature, of the exoproteins produced, influenced the mutant phenotype. Whether the secretion defect described here resides in a *B. subtilis* homologue of a known secretion locus, or in a novel factor, must await mapping and characterization of the relevant genetic locus, as discussed above.

Judging from the small number, and nature, of secretion mutants that have been identified, it seems likely that *B. subtilis* can tolerate only a marginal limitation of its secretion capacity. The *secA* mutant NIG1152 was identified in a screen for division mutants; its secretion defect was revealed in subsequent analyses (Takamatsu *et al.*, 1992). In our hands, no scorable difference from wt was observed with the *secA/div-341* mutant in plate assays for either protease or α -amylase secretion at the 'semi-permissive' temperature. Moreover, localized mutagenesis of the *B. subtilis* *secY* gene, whose *E. coli* homologue encodes an essential component of the secretion apparatus, produced only sporulation mutants (Yoshikawa *et al.*, 1993), with no detectable secretion defects (Yoshikawa *et al.*, 1993; R. Freudl, personal communication). It may be significant that like these *secY* mutants and *secA/div-341*, sporulation of MFJ566 was depressed (13% vs wt 57%).

We have demonstrated that the indirect procedure of screening for raised LacZ activity of a secretory LacZ

fusion protein can indeed reveal mutants with secretory defects in *B. subtilis*. A thorough understanding of the *Bacillus* secretion process will come from a broadly based approach involving genetic screening systems, such as the one described here, to supplement the search for homologues of known secretion loci, and the development of *in vitro* translocation methods. These should reveal whether the remarkable capacity of the *Bacillus* spp. to secrete proteins is dependent on any factors unique to these Gram-positive organisms.

NOTE ADDED IN PROOF

Snyder & Silhavy (1995) have recently shown that LacZ fusion proteins secreted to the periplasm of *E. coli* can have a toxic effect quite distinct from lethality associated with jamming of the secretory machinery.

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