Sporulation of a Bacitracin-sensitive Mutant of *Bacillus licheniformis* Is Self-inhibited by Bacitracin

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(Received 2 May 1989; revised 3 July 1989; accepted 7 July 1989)

A mutant of *Bacillus licheniformis* (BLU166) sensitive to its own antibiotic bacitracin was isolated and the mutation *bcr-1* was mapped close to the bacitracin synthetase genes. The sensitivity was shown to be specific for bacitracin. Two further bacitracin-sensitive strains were constructed, one (BLU171) with normal ability to synthesize bacitracin, and one (BLU170) a bacitracin non-producer. In addition to an increased sensitivity of growing cells to bacitracin, sporulation of the mutant strain BLU171 was self-inhibited by bacitracin. It is concluded that (1) there might exist at least two levels of resistance to bacitracin; (2) mutation *bcr-1* affects a 'structural' component, which may protect the sensitive reaction of cell-wall biosynthesis; (3) sporulation is affected to a greater extent by bacitracin than vegetative growth; and (4) synthesis of bacitracin is independent of the presence of this resistance mechanism since the sensitive mutant produces similar amounts of the antibiotic to the wild-type strain.

INTRODUCTION

The late stages of growth in the bacilli are characterized by sporulation, synthesis of secondary metabolites and the eventual response of cells to the latter (Schaeffer, 1969). A close relationship between cessation of vegetative growth and synthesis of antibiotics is supported by observations of sensitivity of producer organisms to their own antibiotic. This sensitivity appears in the absence of antibiotic synthesis, i.e. during vegetative cell growth (Jayaraman & Kannan, 1972; Schmitt & Freese, 1968; Snoke & Cornell, 1965). These observations could indicate that peptide antibiotics actually inhibit growth and allow initiation of sporulation (Jayaraman & Kannan, 1972; Sarkar *et al.*, 1977). In addition, peptide antibiotics may have regulatory functions in sporulation (Federn & Ristow, 1987; Fisher & Blumenthal, 1982; Katz & Demain, 1977) or in the outgrowth of germinating spores (Daher *et al.*, 1985). However, mutants defective in antibiotic biosynthesis did not lose the ability to sporulate, thus ruling out a direct involvement of these peptides in the sporulation process (Haavik & Thomassen, 1973; Piret & Demain, 1983).

Studies of the relationship between synthesis of antibiotics and the resistance of cells to their own antibiotics are additionally complicated by the involvement of sporulation processes in biosynthesis of antibiotics. While it seems that mutations affecting enzymes involved in antibiotic synthesis have no direct effect on sporulation, mutations in genes responsible for sporulation impair both antibiotic biosynthesis and resistance to the antibiotic. This is the principal characteristic of stage 0 (*spo0*) sporulation mutations, which block early stages of sporulation (Piggot & Coote, 1976).

The mechanism whereby cells become resistant to their own peptide antibiotics is unknown. At least two possible mechanisms of neutralization have been suggested. First, edein (Kurylo-Borowska & Szer, 1976) and polymyxin (Vasanthan *et al.*, 1980) bind to their respective synthetases; and second, gramicidin S is inactivated by a specific protease (Kurotsu *et al.*, 1982).

The aim of our work was to separate three phenomena in the life of *Bacillus licheniformis*:

Table 1. *Strains of Bacillus licheniformis*

Strain	Genotype	Source
ATCC 10716 UM12	Prototroph, cured of phage LP-52	C. B. Thorne
BLU117	<i>lys-4 bac-54</i>	Podlesek & Grabnar (1987)
BLU166	<i>lys-4 bac-54 bcr-1</i>	This work
ATCC 10716	Prototroph	C. B. Thorne
BLU169	<i>trp-1</i>	This laboratory
BLU170	<i>lys-4 bac-54 bcr-1</i>	This work
BLU171	<i>lys-4 bcr-1</i>	This work
BLU172*	<i>lys-4</i>	This work

* Bacitracin-resistant revertant of mutation *bcr-1*.

sporulation, synthesis of the peptide antibiotic bacitracin, and resistance to bacitracin. A bacitracin-sensitive mutant was isolated and a novel connection between sporulation and synthesis of the antibiotic was found, since sporulation became hypersensitive to bacitracin. Thus, in addition to the proposed role in promotion of sporulation, peptide antibiotics may also inhibit sporulation in a similar way as they inhibit vegetative growth.

METHODS

Bacterial strains. *B. licheniformis* strains are listed in Table 1.

Bacteriophage. The generalized transducing phage SP-15 was propagated and assayed by the method of Taylor & Thorne (1963).

Media and growth conditions. PA medium (Thorne, 1962) contained 8 g nutrient broth (Difco), 5 g NaCl, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.05 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ in 1 litre of deionized water, adjusted to pH 6.0 with NaOH. For transduction tests, M1E minimal medium (McCuen & Thorne, 1971) was used and the cells were incubated at 37 °C.

Assay of sensitivity to antibiotics. Sensitivity of mutants to antibiotics was determined in two ways. The first test was the standard diffusion assay based on inhibition zones in Antibiotic Medium 3 (Difco). In the second test, the culture was grown in PA medium at 37 °C to an OD_{550} value of 0.3 (approximately 8×10^7 cells ml^{-1}). The culture was then divided into an appropriate number of flasks (see below), followed by addition of varying concentrations of antibiotic. Subsequent growth was followed by measuring the OD_{550} with an Uvikon 850 spectrophotometer. The antibiotics tested were as follows (concentrations in parentheses): bacitracin (1–10 i.u. ml^{-1} , increasing stepwise by 0.5 i.u. ml^{-1} , for the bacitracin-sensitive mutants; 10–100 i.u. ml^{-1} , increasing stepwise by 5 i.u. ml^{-1} , for the wild-type); penicillin G (500–2000 i.u. ml^{-1} , increasing stepwise by 250 i.u. ml^{-1}); polymyxin B (5.0–20.0 $\mu\text{g ml}^{-1}$, increasing stepwise by 5 $\mu\text{g ml}^{-1}$); tunicamycin (1.0–10.0 $\mu\text{g ml}^{-1}$, increasing stepwise by 1.5 $\mu\text{g ml}^{-1}$); gramicidin S (1.75–2.75 $\mu\text{g ml}^{-1}$, increasing stepwise by 0.25 $\mu\text{g ml}^{-1}$); vancomycin (0.3–0.6 $\mu\text{g ml}^{-1}$, increasing stepwise by 0.1 $\mu\text{g ml}^{-1}$); and novobiocin (10–100 $\mu\text{g ml}^{-1}$, increasing stepwise by 15 $\mu\text{g ml}^{-1}$). All antibiotics were obtained from Sigma.

Transduction. Transduction tests were done according to Rogolsky (1970). Transduction mixtures were plated on M1E agar with appropriate supplements added to allow recovery of transductants carrying unselected markers. Transductants were analysed after 48 h incubation at 37 °C. Transductant colonies were transferred to PA agar with a lawn of *Micrococcus flavus* and analysed for the Bac phenotype after 24 h incubation at 37 °C.

Mutagenesis. Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was done by the method of Imanaka *et al.* (1981), except that BHI medium (Difco) was used for growth of the culture. Mutagenized bacteria were spread onto Nutrient Broth (NB; Difco) agar plates. After 18 h incubation they were replica-plated onto NB plates with and without 2 i.u. bacitracin ml^{-1} .

Assay of bacitracin. The extracellular level of bacitracin was determined by the method of Haavik & Thomassen (1973). Biosynthesis of bacitracin by mutant non-producing cells in PA medium was below the limit of detection (<5% of the wild-type level).

Determination of sporulation. Heat resistance was determined by heating 0.5 ml samples at 80 °C for 10 min. The extent of sporulation was determined by diluting the treated culture in sterile deionized water and spreading samples on NB plates.

RESULTS

For isolation of the bacitracin-sensitive mutant of *Bacillus licheniformis*, we used strain BLU117, which has a defect in bacitracin synthetase (Podlesek & Grabnar, 1987), so as to avoid

Fig. 1. Induction of bacitracin resistance in the wild-type (ATCC 10716) and in the bacitracin-sensitive strain BLU170 of *B. licheniformis*. Antibiotic was added at time zero, when the cultures were in the mid-exponential phase of growth. Wild type: $\square$, control; $\circ$, plus 50 i.u. bacitracin ml^{-1} ; $\triangle$, plus 50 i.u. bacitracin ml^{-1} added to a culture pre-grown in the presence of 0.5 i.u. bacitracin ml^{-1} . BLU170: $\blacksquare$, control; $\bullet$, plus 5 i.u. bacitracin ml^{-1} ; $\blacktriangle$, plus 5 i.u. bacitracin ml^{-1} to a culture pre-grown in the presence of 0.5 i.u. bacitracin ml^{-1} .

Table 2. Analysis of *bac-54*, *bcr-1* and *lys-4* loci by three-factor transduction crosses

The recipient was strain BLU166 (*lys-4 bcr-1 bac-54*), and the donor was the wild-type strain ATCC 10716.

Selection	Recombinant classes*			No. of clones
	Lys	Bcr	Bac	
Lys ⁺	1	0	0	200
	1	0	1	5
	1	1	0	36
	1	1	1	384
Bcr ⁺	0	1	0	9
	0	1	1	35
	1	1	0	13
	1	1	1	156

* '0' and '1' refer to recipient and donor markers respectively.

possible lethal consequences of simultaneous bacitracin synthesis. The mutation *bcr-1* in the bacitracin-sensitive mutant BLU166 was mapped by three-factor transduction crosses with phage SP-15 (Table 2). The results indicated that the mutation is located in the vicinity of the *bac* (bacitracin synthetase genes) and *lys* loci. Results of three-factor transductional crosses suggested the following order of loci: *bac-54-bcr-1-lys-4*.

Strains BLU170 (sensitive to bacitracin and bacitracin non-producer) and BLU171 (sensitive to bacitracin and bacitracin producer) were constructed by transduction involving BLU166 as donor and BLU169 as recipient.

The effect of bacitracin on the growth kinetics of the bacitracin-sensitive strain BLU170 is shown in Fig. 1. It can be seen that resistance to its own antibiotic is significantly reduced in strain BLU170. On the other hand, induction of resistance (Yaroslavtseva *et al.*, 1981), achieved by previous growth in medium containing 0.5 i.u. bacitracin ml^{-1} , was similar in the mutant and wild-type strains.

In order to establish the specificity of sensitivity to bacitracin, the sensitivity of strain BLU170 to other antibiotics that inhibit cell wall biosynthesis or destroy the integrity of the membrane was evaluated as described in Methods. No difference in sensitivity between the wild-type (ATCC 10716) and bacitracin-sensitive strain BLU170 could be detected for any of

Fig. 2. Sporulation and synthesis of bacitracin by *B. licheniformis* after the end of exponential growth (time zero) in PA medium. Sporulation: □, wild-type (ATCC 10716); ▲, BLU170; ■, BLU171. Bacitracin synthesis: ○, wild-type; ●, BLU171. Note the difference in the sporulation of BLU171 and wild-type cells: sporulation of BLU171 is inhibited at 5–6 h after the end of exponential growth and this inhibition coincides with the peak of bacitracin synthesis.

Fig. 3. Inhibition of sporulation upon addition of bacitracin at t_2 to *B. licheniformis* strain BLU170: □, control (no bacitracin added); ○, plus 1.0 i.u. bacitracin ml^{-1} ; ●, plus 2.0 i.u. bacitracin ml^{-1} . ■, Wild-type plus 50 i.u. bacitracin ml^{-1} .

the antibiotics tested (penicillin G, D-cycloserine, vancomycin, gramicidin S, polymyxin B, tunicamycin and novobiocin; data not shown).

Sporulation of strains BLU170, BLU171 and the wild-type was also compared to eliminate the possibility of a *spoD* mutation, which in *B. subtilis* also results in an increased sensitivity to its own peptide antibiotic and to polymyxin B (Piggot & Coote, 1976). The final number of heat-resistant spores after 30 h incubation at 37 °C was similar in all strains (Fig. 2). On the other hand, a striking difference in the course of differentiation was observed (Fig. 2). Approximately 5–6 h after the cessation of exponential growth (t_0), formation of spores in strain BLU171 was inhibited, although the number of vegetative cells was similar to the wild-type strain. Sporulation proceeded after a delay of a few hours, forming a typical biphasic growth curve. The start of inhibition of sporulation coincided with the peak of bacitracin synthesis (Fig. 2). The sporulation pattern of strain BLU170 was similar to that of the wild-type (Fig. 2), as was that of strain BLU172, a spontaneous bacitracin-resistant revertant of BLU171 (data not shown).

To establish the role of bacitracin in inhibition of sporulation, bacitracin was added to cultures of the sensitive nonproducer BLU170 at the time (t_2) at which bacitracin synthesis commences in wild-type cultures (Fig. 3). Under these conditions sporulation of BLU170 did not differ significantly from that of BLU171. The concentration of antibiotic used was the same as is found in the supernatant of cultures of wild-type cells. About 50-fold greater concentrations were required to partly inhibit sporulation of the wild-type strain (Fig. 3).

It is known from inhibition studies with bacitracin and vancomycin in *B. subtilis* that in stage I of sporulation transient cell-wall synthesis is required to support septum formation (Dancer, 1979). Both antibiotics affect sporulation by inhibiting cell-wall biosynthesis. In contrast to the effect of bacitracin, the effect of vancomycin on sporulation of both BLU170 and the wild-type strains of *B. licheniformis* is similar (data not shown). Thus the sensitivity of sporulation processes in the mutant strains BLU170 and BLU171 is restricted solely to bacitracin.

DISCUSSION

The proximity of the *bcr-1* locus to the bacitracin synthetase genes does not establish that the product of the *bcr-1* locus is responsible for bacitracin resistance in *B. licheniformis*, although many genes involved in antibiotic synthesis are linked to corresponding genes for resistance. In addition, this region of the chromosome contains many genes involved in the sporulation process. Some of them (*spo0*) are pleiotropic in nature (Piggot & Coote, 1976). It is characteristic that polymyxin B, known to act upon the membrane (Storm *et al.*, 1977) affects *spo0* mutants to a greater extent than *spo*⁺ strains (Guespin-Michel, 1971).

By showing that the *bcr-1* mutation does not increase sensitivity to any other antibiotic tested it is possible to exclude the possibility that it is a *spo0* type of mutation. On the other hand, analysis of sporulation in strains with defective and normal bacitracin synthetase (BLU171 and BLU170 respectively), both harbouring a *bcr-1* mutation, demonstrated self-inhibition by bacitracin in strain BLU171. From these results we conclude the following.

1. In *B. licheniformis* at least two levels of resistance to bacitracin exist: (a) an inducible one, and (b) a 'structural' one. The mutation *bcr-1* affects the 'structural' resistance to bacitracin. This gene maintains the basal level of resistance and its activity seems to be independent of the growth stage. On the other hand it could be that the *bcr-1* mutation does not completely inactivate the inducible resistance mechanism. However, this possibility seems unlikely because the kinetics of induction of resistance to bacitracin is similar in mutant BLU171 and the wild-type (Fig. 1).

2. Because of the specificity of sensitivity caused by the *bcr-1* mutation it is unlikely that this is a consequence of gross changes in permeability of the cell wall. Because of the inhibition of cell wall biosynthesis caused by bacitracin, it is probable that specific protection of the sensitive reaction, namely dephosphorylation of the undecaprenyl phosphate carrier in the membrane (Storm, 1974), is affected. On the other hand, by analogy with gramicidin S in *B. brevis* (Kurotsu *et al.*, 1982), there is a possibility that a sporulation-related intracellular protease could degrade bacitracin. However, sensitivity of the mutant strain is also increased during vegetative growth, which precedes appearance of the protease in *B. licheniformis* (Vitković & Sadoff, 1977).

3. Paradoxically, sporulation processes are essentially more sensitive to bacitracin than vegetative growth, even though the onset of bacitracin biosynthesis and the sporulation processes coincide. Considering the number of cells present upon addition of bacitracin, sporulation of BLU170 is inhibited by a concentration of antibiotic an order of magnitude lower than that required to inhibit vegetative growth. Thus this mode of resistance might primarily protect this stage of the *B. licheniformis* life cycle.

4. Synthesis of bacitracin is independent of resistance to it. In spite of its sensitivity, mutant BLU171 synthesizes similar levels of antibiotic as wild-type cells. Genetically distinct mechanisms that might control both processes were also suggested by Guespin-Michel (1971) on the ground that some partial *spo0* revertants of *B. subtilis*, isolated as cells resistant to their own antibiotic, could not resume antibiotic synthesis. However, because of the pleiotropy of *spo0* mutations and the possibility of secondary mutations (e.g. in permeability), unrelated to sporulation, this suggestion was inconclusive.

On the grounds of sporulation inhibition, it can be speculated that in nature bacitracin might act upon competitive spore-forming bacilli, preventing or impeding their sporulation.

We are grateful to M. Janc for critical reading of the manuscript. This work was supported by the Research Council of Slovenia and 'Krka'.

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