Enhanced Secretion of *Escherichia coli* β -Lactamase by a Spontaneous Erythromycin-resistant Mutant of *Bacillus subtilis*

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(Received 5 July 1988; revised 14 November 1988; accepted 8 December 1988)

An extracellular-protease-deficient mutant, ME142, was isolated from *Bacillus subtilis* as a spontaneous erythromycin-resistant (Ery^r) clone. This mutant showed conditional sporulation and only sporulated normally in the absence of erythromycin. In the presence of the antibiotic, sporulation was greatly reduced. Production of extracellular proteases by ME142 also exhibited conditional deficiency, possibly due to pleiotropic effects of the sporulation deficiency. The production of protease was 2–10% that of the wild-type level in the presence of erythromycin. ME142 showed poor competence for transformation even in the absence of erythromycin; however, derivatives of ME142 were isolated which had the same Ery^r phenotype but which exhibited normal competence. One such mutant, ME162, was used as a host for the secretion of *Escherichia coli* β -lactamase. The amount of β -lactamase in the culture supernatants of ME162 increased significantly when the cells were cultured with erythromycin, suggesting that proteolysis of the β -lactamase in the supernatants of ME162 was greatly reduced as compared to that in the supernatants of the wild-type strain.

INTRODUCTION

Among mutants of *Bacillus subtilis* showing resistance to several antibiotics are some that have been shown to be asporogeneous (Piggot & Coote, 1976). Some erythromycin-resistant (Ery^r) mutants for example, show conditional sporulation, exhibiting a temperature-sensitive sporulation (Spo^{ts}) phenotype or sporulating only in the absence of erythromycin (Sharrock *et al.*, 1981). An Ery^rSpo^{ts} strain obtained by mutagenesis with ethyl methane sulphonate failed to sporulate at a high temperature (47 °C) irrespective of the presence of erythromycin, but sporulated normally at 30–35 °C (Goldman & Tipper, 1979). Domoto *et al.* (1975) reported a spontaneous Ery^r mutant (*ery1040*) which grew normally in the presence or absence of erythromycin but sporulated only in its absence. The fact that erythromycin affects the 50S ribosomal subunit and that Ery^r mutants have an altered ribosomal protein L-17 suggests a possible involvement of translation in the regulation of sporulation (Sharrock *et al.*, 1981).

Recent advances in the ability to manipulate genes in *B. subtilis*, and its capacity to secrete large amounts of protein into the growth medium, have led to this organism being used as a host for the synthesis and secretion of foreign proteins. A number of groups have reported the construction of secretion vector systems utilizing genes for *Bacillus* extracellular enzymes such as α -amylase, penicillinase and protease (Chang *et al.*, 1983; Palva *et al.*, 1983; Honjo *et al.*, 1985; Shiroza *et al.*, 1985). However, in all cases, the yields of foreign gene products recovered from the culture medium were less than 10% of the original enzyme level. The main reason for the reduction in productivity seems to be degradation by the extracellular alkaline and neutral proteases (Shiroza *et al.*, 1985; Ulmanen *et al.*, 1985). Accordingly, extracellular-protease-deficient mutants have been isolated to overcome this problem. Recently, some investigators have reported success in secreting foreign proteins by use of protease-deficient mutants carrying

lesions in the structural genes of proteases (Nakamura *et al.*, 1985; Wong *et al.*, 1986; Fahnestock & Fischer, 1987; Honjo *et al.*, 1987) or by using a *spo0A* mutant (Fahnestock & Fischer, 1986).

In this paper, we describe the isolation of a spontaneously occurring Ery^r mutant which sporulates only in the absence of erythromycin. Since this mutant produces less than 10% of the extracellular protease activity of the wild-type strain in the presence of erythromycin, we have used it to examine the production and stability of *Escherichia coli* β -lactamase. We discuss the potential value of this mutant as a host for the production of foreign proteins in *B. subtilis*.

METHODS

Bacterial strains and plasmids. Bacterial strains used in this study are listed in Table 1. Plasmids pNYB3, for the extracellular production of β -lactamase, and pNZ2, for the intracellular production of β -galactosidase, are shown in Fig. 1 (a, b). Plasmid pNYB3 was constructed as follows. A synthetic oligonucleotide (Fig. 1 c), containing both a ribosome-binding site and the signal peptide coding region of the *Bacillus amyloliquefaciens* α -amylase gene (Palva *et al.*, 1982) was fused in-frame to the *E. coli* β -lactamase structural gene (*bla*) lacking the signal sequence (Palva *et al.*, 1982). This fused fragment was introduced into the *Eco*RI and *Hind*III sites of the *B. subtilis* vector pNC601 (Kamakura *et al.*, 1987). Plasmids pNZ2 and pUB110 were described previously (Nagami & Tanaka, 1986; McKenzie *et al.*, 1986).

Media. The media used in this study were 2 $\times$ SG medium (Leighton & Doi, 1971), LB agar, and minimal agar consisting of Spizizen's minimal medium (Spizizen, 1958) supplemented with Casamino acids (0.5%), tryptophan (50 μ g ml⁻¹) and agar (1.5%, w/v). Trimethoprim, erythromycin and kanamycin were added to a final concentration of 1, 2 and 5 μ g ml⁻¹, respectively, unless otherwise stated.

Growth and sporulation. Cells were cultured in 2 $\times$ SG medium at 37 °C for 24 h with shaking. Heat-resistant spores and viable cells were counted by plating on to LB agar plates with or without heat treatment at 80 °C for 10 min. Growth was monitored in a Klett-Summerson colorimeter equipped with a no. 66 red filter.

Transformation. Transformation of *B. subtilis* competent cells with plasmid DNA was performed as described previously (Tanaka & Kawano, 1980). Transformants were selected on Spizizen agar plates containing either kanamycin (for pUB110) or trimethoprim (for pNC601).

Enzyme assay. Activities of protease in the culture supernatants, β -galactosidase in the cell extracts and β -lactamase in both supernatants and extracts were assayed as described previously (Nagami & Tanaka, 1986; Tanaka *et al.*, 1987).

RESULTS

Isolation and characterization of Ery^r mutants

Spontaneous Ery^r mutants of *B. subtilis* MT142 were selected on Spizizen agar plates containing erythromycin (2 μ g ml⁻¹) at 37 °C. The frequency of mutation was about 4.3×10^{-8} per colony-forming unit. One of these mutants was designated as ME142 and used for further study. The ability of ME142 to sporulate was reduced only slightly in the absence of erythromycin, but was greatly reduced in its presence (Table 2). All of the resulting heat-resistant spores of ME142 obtained in the absence of erythromycin exhibited erythromycin resistance, indicating that they were not revertants. These results demonstrate that ME142 belongs to the *ery1040* type of conditional asporogeneous mutant reported by Domoto *et al.* (1975). In the absence of erythromycin, the growth pattern of ME142 was similar to that of MT142. In the presence of erythromycin, however, ME142 grew at the same rate as MT142 until the early stationary phase, but the final cell density of ME142 was slightly less than that of MT142.

Since the appearance of extracellular proteases is closely associated with the process of sporulation (Piggot & Coote, 1976), we examined the activity of extracellular proteases in ME142 (Table 3). When ME142 was grown with erythromycin, the protease activities in the culture supernatants during the stationary phase (12, 24, 48 h) were only 2–10% of those found for MT142. In the absence of erythromycin however, the protease activities of MT142 and ME142 were comparable. The protease level of the neutral-protease-deficient mutant MT-2 (Fujii *et al.*, 1983) at 12 h was similar to that found for ME142 in the presence of erythromycin, but increased about 5–20-fold with increasing culture time (24, 48 h), possibly due to the production of alkaline protease. These results suggest that ME142 could be a more effective protease-deficient mutant than MT-2. Furthermore, these results clearly show that extracellular production of proteases is closely associated with sporulation.

Fig. 1. Physical maps of the expression plasmids pNYB3 and pNZ2. The letters in parentheses indicate the recognition sites which were cleaved with the respective enzymes, filled in and blunt-end ligated. *bla*, *dfrA*, *lacZ* and *thyB* designate the *E. coli* β -lactamase (ampicillin resistance), *B. subtilis* Tmp^r-dihydrofolate reductase, *E. coli* β -galactosidase and *B. subtilis* thymidylate synthase B genes, respectively. (a) pNYB3. The filled box shows the *bla* gene that lacks its own signal sequence. The open box indicates the synthetic oligonucleotide (117 bp) whose nucleotide sequence is shown in (c). The ribosome-binding site (RBS) and the *B. amyloliquefaciens* α -amylase signal sequence are shown by the dotted region. The thin line represents the vector region of pNC601. The internal arrow shows the direction of translation. (b) pNZ2. The filled box shows the *lacZ* gene lacking the first eight codons of the amino-terminal end. The open box indicates the *prtR* gene (Nagami & Tanaka, 1986), whose open reading frame (amino acids 1–26) is shown by the dotted region. The thin and thick lines represent the vector regions of pNC6 (Nagami & Tanaka, 1986) and pBR322, respectively. The internal arrow indicates the direction of translation. (c) The nucleotide sequence of the synthetic oligonucleotide in pNYB3. The deduced amino acid sequence (one-letter symbols) is also shown. The underline indicates the RBS. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sma*I; V, *Eco*RV.

Table 1. *Bacillus subtilis* strains used in this study

Strain	Properties*	Origin or reference
MT142	<i>arg-15 leuB8 trpE thyA</i> ; Tmp ^r	This study
ME142	<i>arg-15 leuB8 trpE thyA</i> ; Tmp ^r Ery ^r	This study
ME152	<i>arg-15 leuB8 thyA</i> ; Tmp ^r Ery ^r	This study (MI112 DNA $\rightarrow$ ME142)
ME162	<i>arg-15 leuB8</i> ; Ery ^r	This study (ME152 DNA $\rightarrow$ RM125)
MI112	<i>arg-15 leuB8</i> ; <i>thr5 recE4 hsmM hsrM</i>	Tanaka & Kawano (1980)
RM125	<i>arg-15 leuB8 hsmM hsrM</i>	Uozumi <i>et al.</i> (1977)
MT-2	<i>leuC7 trpC2 hsmM hsrM</i> ; Npr ⁻	Fujii <i>et al.</i> (1983)

* Tmp^r, trimethoprim resistance; Ery^r, erythromycin resistance; Npr⁻, deficiency of extracellular neutral protease.

Table 2. *Sporulation frequency of MT142 and Ery^r mutants*

Strain	Addition of erythromycin	No. of viable cells ml ⁻¹	No. of spores ml ⁻¹ *	Sporulation frequency (%)
MT142	—	8.6 × 10 ⁸	5.0 × 10 ⁸	58
ME142	—	3.5 × 10 ⁸	1.1 × 10 ⁸	32
ME142	+	5.3 × 10 ⁸	3.9 × 10 ⁵	0.072
ME152	—	6.5 × 10 ⁸	5.6 × 10 ⁸	86
ME152	+	2.7 × 10 ⁸	6.4 × 10 ⁴	0.023
ME162	—	4.3 × 10 ⁸	2.6 × 10 ⁸	61
ME162	+	1.7 × 10 ⁸	9.1 × 10 ⁴	0.053

* Sporulation was examined after 24 h incubation with or without erythromycin (2 µg ml⁻¹) as described in Methods.

Table 3. *Production of extracellular proteases by MT142 and Ery^r mutants*

Strain	Addition of erythromycin	Protease activity [U (ml supernatant) ⁻¹]*		
		12 h	24 h	48 h
MT142	—	0.18	0.61	0.91
ME142	—	0.034	0.21	0.41
ME142	+	0.019	0.034	0.014
ME152	—	0.024	0.17	0.31
ME152	+	0.025	0.069	0.068
ME162	—	0.039	0.36	0.35
ME162	+	0.016	0.020	0.010
MI112	—	0.022	0.46	0.55
MT-2	—	0.057	0.15	0.30

* Each strain was cultured in 2 × SG medium with or without erythromycin at 37 °C for 12, 24 or 48 h. Viable cell numbers were 2 × 10⁸ to 8 × 10⁸ per ml of culture. Cell numbers of each Ery^r mutant cultured with and without erythromycin were similar. The culture supernatants were assayed as described in Methods.

Many *spo0* mutants are incapable of developing competence, exceptions being *spo0C*, *spo0G* and *spo0J* mutants (Sadaie & Kada, 1983). We therefore investigated the transformation capacity of ME142 using plasmid pUB110 (Table 4). ME142 showed poor competence (only 3% of the MT142 level), even under conditions when the cells were sporulation-proficient, i.e. without the addition of erythromycin.

Development of an Ery^r-associated protease-deficient mutant with normal competence

The poor competence-development ability of ME142 would be a disadvantage for applications in which this strain was to be used as a host for molecular cloning. In order to isolate an Ery^r mutant with normal competence, chromosomal DNA from MI112, which exhibits normal competence, was introduced into ME142 by selecting for an unlinked *trp*⁺ marker. An Ery^r mutant, ME152, was isolated which had recovered the normal frequency of pUB110-mediated transformation, but which retained a similar growth rate to that of ME142, and the conditional sporulation and protease deficiency characteristic of ME142 (Tables 2 and 3). These results indicate that the poor competence shown by ME142 is apparently independent of erythromycin resistance.

We have previously developed plasmids such as pTL12 (Tanaka & Kawano, 1980), pNC601 and pNC602 (Kamakura *et al.*, 1987) as cloning vectors carrying a *Tmp*^r selection marker. Strains ME142, ME152 and MT142 were, however, resistant to trimethoprim (1 µg ml⁻¹; Table 4) because of their *thyA thyB*⁺ genetic background (Neuhard *et al.*, 1978). In order to use these vectors in the Ery^r mutants, we further constructed an Ery^r strain carrying *thyA*⁺ *thyB*⁺. By transforming ME152 DNA into competent cells of RM125, several Ery^r transformants were

Table 4. Transformation frequency of MT142 and *Ery^r* mutants

Recipient strain	Selective marker*	No. of viable cells ml ⁻¹	No. of transformants ml ⁻¹	Transformation frequency
MT142	Km ^r	1.4×10^8	2.3×10^5	1.7×10^{-3}
ME142	Km ^r	1.7×10^8	6.5×10^3	3.8×10^{-5}
ME152	Km ^r	4.4×10^8	2.4×10^5	5.5×10^{-4}
ME162	Km ^r	6.6×10^7	7.4×10^4	1.1×10^{-3}
MI112	Km ^r	4.7×10^7	2.2×10^5	4.6×10^{-3}
MT142	Tmp ^r	1.4×10^8	ND	—
ME142	Tmp ^r	1.7×10^8	ND	—
ME152	Tmp ^r	4.4×10^8	ND	—
ME162	Tmp ^r	6.6×10^7	4.9×10^4 †	7.4×10^{-4} †
MI112	Tmp ^r	4.7×10^7	5.9×10^4	1.3×10^{-3}

ND, not determined. MT142, ME142 and ME152 were Tmp^r because of their *thyA* genotype (Neuhard *et al.*, 1978).

* pUB110 and pNC601 DNA (0.23 and 1.1 μ g ml⁻¹) were used to transform the cells to kanamycin resistance (Km^r) and trimethoprim resistance (Tmp^r), respectively.

† Tmp^r transformants of ME162 were selected on Spizizen agar supplemented with trimethoprim (10 μ g ml⁻¹).

obtained. One such transformant, ME162, was resistant to 1 μ g trimethoprim ml⁻¹, but not to 10 μ g ml⁻¹. This strain showed normal competence for pNC601- and pUB110-mediated transformation (Table 4), and, like ME142 and ME152, was conditional for both the sporulation- and the protease-deficiency (Tables 2 and 3). This strain also showed a similar growth pattern to those of ME142 and ME152.

Extracellular production of *E. coli* β -lactamase by *B. subtilis* ME162

In order to test the usefulness of the protease-deficient phenotype of mutant ME162, we studied the secretion of the *E. coli* β -lactamase by this strain. A recombinant plasmid pNYB3 was constructed and used for the production of β -lactamase as described in Methods and Fig. 1. The *bla* gene is transcribed from the promoter for the upstream *B. subtilis* *dfr* operon consisting of genes *thyB* and *dfrA* (Iwakura *et al.*, 1988; Nagami *et al.*, 1988), and translated from the synthetic ribosome-binding site (Fig. 1c). The β -lactamase is secreted with the aid of the α -amylase signal peptide. Since the *dfr* promoter is a vegetative-type promoter (Iwakura *et al.*, 1988), it functions in sporulation-deficient cells such as ME162.

ME162 and the wild-type strain, MI112, both carrying pNYB3, were grown in 2 $\times$ SG medium containing antibiotics (trimethoprim) and erythromycin for ME162, or trimethoprim for MI112), and the β -lactamase activities in the culture supernatants and in the sonicated cells were measured after different periods of culture (Fig. 2). Both strains secreted more than 90% of β -lactamase into the culture supernatants, and their maximum activities were similar (about 8 U ml⁻¹) at the beginning of the stationary phase (12 h). β -Lactamase activity in ME162 declined slightly during further culture, whereas that in MI112 declined drastically. At 24 h, most of the activity in MI112 had disappeared, whereas in ME162 more than 70% of the maximum level remained. Under identical conditions, the host cells carrying the vector, pNC601, had no detectable β -lactamase activity. These results show that in 24 h cultures extracellular β -lactamase was about fivefold more stable in ME162 compared with that in MI112. The production of extracellular protease by MI112 increased throughout cultivation, whilst the levels produced by ME162 remained virtually unaltered; after 24 h, MI112 secreted about 10 times as much protease as did ME162 (Fig. 2).

In order to determine the influence of extracellular protease on the stability of β -lactamase, equal volumes of the culture supernatants from 12 h and 20 h cultures were mixed, and the levels of β -lactamase activity were determined (Fig. 3). When the supernatants were obtained from MI112, the β -lactamase activity had decreased to about half the initial level after 0.5 h, and had disappeared after 2 h of incubation. In contrast, the β -lactamase activity in the supernatants

Fig. 2. Extracellular production of *E. coli* β -lactamase by *B. subtilis* ME162 (a) and MI112 (b), both carrying pNYB3. Cells were cultured at 37 °C in 2 $\times$ SG medium (0.5% inoculum) containing either trimethoprim and erythromycin for ME162 or trimethoprim for MI112. $\circ$, β -Lactamase activity in culture supernatants; $\bullet$, β -lactamase activity in sonicated cells; $\square$, protease activity in the culture supernatants; $\blacksquare$, growth monitored in a Klett-Summerson colorimeter.

obtained from ME162 declined at a slower rate, with approximately 75% of the initial level remaining after 3 h incubation. These results indicate that the proteolysis of β -lactamase in the supernatants of ME162 was greatly reduced as compared to that in the supernatants of the wild-type strain.

In spite of the protease deficiency in ME162, the amount of β -lactamase did not increase after 12 h as it did in a mutant with lesions in both the alkaline and neutral protease structural genes (Nakamura *et al.*, 1985; Wong *et al.*, 1986; Honjo *et al.*, 1987). In order to understand the reasons for this difference in behaviour, we measured the intracellular β -galactosidase activity transcribed from the *dfr* promoter in both ME162 and MI112 carrying pNZ2. Plasmid pNZ2 contains a fused gene consisting of the coding region of the *B. subtilis prtR* (amino acids 1–26) and the *E. coli lacZ* gene (amino acids 9–1023) downstream of the *dfr* operon (Fig. 1b; Nagami & Tanaka, 1986). Accordingly, the *lacZ* gene is transcribed and translated by the same *dfr* polycistronic expression system (Nagami *et al.*, 1988) as in the case of β -lactamase production (Fig. 1). In this construct, the production of β -galactosidase reflects the activity of the *dfr* polycistronic expression system, irrespective of the influence of extracellular proteases. As shown in Fig. 4, the intracellular β -galactosidase activity in ME162 increased during exponential growth, reached a maximum in early stationary phase and remained at the same level during the stationary phase. Host cells carrying the vector, pNC61 (Nagami & Tanaka, 1986), did not show β -galactosidase activity under the conditions that we used. This result shows that the synthesis of proteins driven by the *dfr* promoter is lowered during stationary phase as compared to that in the exponential phase. The profile of β -galactosidase production was similar in MI112, although the maximum activity was only half that found in ME162 (Fig. 4). The reason for this difference is not known. Proteolytic degradation could not be ruled out as the reason for the limited secretion, since a small amount of protease activity was detectable in the culture supernatants of ME162 (Fig. 2, Table 3) and the β -lactamase activity in the mixture of supernatants in ME162 decreased gradually during incubation (Fig. 3). These results suggest that the limitation of β -lactamase secretion in ME162 is due to the combined effects of lowered synthesis and proteolytic degradation.

Fig. 3

Fig. 3. Stability of β -lactamase in culture supernatants. Equal volumes (4 ml) of the 12 h and 20 h culture supernatants of each strain were mixed and incubated at 37 °C. β -Lactamase activities of the mixtures obtained from ME162 (○) and MI112 (●) were assayed at the times indicated.

Fig. 4

Fig. 4. Production of *E. coli* β -galactosidase by *B. subtilis* strains ME162 (○) and MI112 (●). β -Galactosidase activity was assayed as described in Methods. Growth of strains ME162 (□) and MI112 (■) was monitored in a Klett-Summerson colorimeter. Culture conditions were the same as those described in the legend to Fig. 2.

DISCUSSION

We have isolated a spontaneous *Ery^r* mutant, *B. subtilis* ME142, which is conditional for sporulation, and produces only 2–10% of the wild-type level of extracellular proteases in the presence of erythromycin. The correlation between these conditional deficiencies suggests that the extracellular production of proteases is closely associated with the process of spore formation, although Yang *et al.* (1984) and Kawamura & Doi (1984) reported that the extracellular proteases were not required for normal sporulation. The phenotype of ME142 is similar to that of a *spo0A* mutant (Sadaie & Kada, 1983; Fahnestock & Fischer, 1986) with respect to the extracellular-protease deficiency and lowered competence for transformation. However, we were able to isolate *Ery^r* mutants, ME152 and ME162, with normal competence. Thus, these *Ery^r* mutants are distinct from the *spo0A* mutant. Furthermore, these results indicate that ME142 carries two mutations, one responsible for the *Ery^r* phenotype and the other conferring lowered competence for transformation.

Our aim was to investigate the possibility of utilizing this pleiotropic and conditional protease-deficient mutant as a host for the extracellular production of foreign proteins. A similar attempt was reported by Fahnestock & Fischer (1986), in which a cloned staphylococcal protein A was produced by *B. subtilis* to a level of 1 g (1 culture)⁻¹ by use of a *spo0A* mutant showing a pleiotropic phenotype including protease deficiency. In the host–vector system described here, a sporulation-deficient host was used in combination with a vegetative-type promoter. The mutant was more effective in protecting β -lactamase from protease attack than was the wild-type (Fig. 2). The yield of β -lactamase by ME162 did not increase with cultivation time, probably because of the lowered synthesis of the enzyme from the *dfp* promoter during stationary phase, and proteolytic degradation (Figs 3 and 4). However, the possibility of inefficient transport and/or processing of the β -lactamase precursor has not been eliminated. Recently, Schein *et al.* (1986) and Saunders *et al.* (1987) demonstrated that significant amounts of

precursors of human interferon- $\alpha 2$ and human serum albumin were cell associated when the signal sequence and promoter of the *B. amyloliquefaciens* α -amylase gene was joined to the coding sequence of the mature forms of these proteins.

Ruppen *et al.* (1986) succeeded in high-level intracellular expression of human growth hormone (1.5 g l^{-1}) by using a *spo0A* mutant of *B. subtilis*. Our experiment also demonstrated that the intracellular production of β -galactosidase in ME162 was twice that in MI12. More recently, Perego & Hoch (1988) reported that the *hpr* gene product, a negative regulator of protease production, is constitutively overproduced in a *spo0A* mutant. It was also found that the *hpr* gene affects the production of the intracellular serine protease in addition to the extracellular proteases (Ruppen *et al.*, 1988). These results may reflect the fact that there are lower activities of intracellular as well as extracellular proteases caused by the overexpression of the *hpr* gene in sporulation-deficient strains such as *spo0A* and Ery^r mutants. Therefore, it might be expected that sporulation-deficient strains in general may be suitable hosts for the intracellular production of foreign proteins by *B. subtilis*.

We thank M. Kawata and M. Takahashi for technical assistance and Y. Teranishi for the support and interest in this work. We are grateful to I. Palva for the gift of the *bla* gene lacking its own signal sequence and S. Aiba for *B. subtilis* MT-2.

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