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Characterization of *Bacillus subtilis* DSM704 and Its Production of 1-Deoxynojirimycin

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A *Bacillus subtilis* strain, DSM704, was characterized by genetic means, and its production of a human intestinal sucrase inhibitor, 1-deoxynojirimycin, was described. Synthesis of this compound is detected concomitant with the detection of heat-resistant spores. The amount of 1-deoxynojirimycin produced is highly dependent on the carbon source, with growth on substrates metabolized via glycolysis giving the greatest amount of production (up to 1 mg/ml). 1-Deoxynojirimycin appears to be nonmetabolizable by the producing strain in that it cannot serve as a sole carbon or nitrogen source.

At the onset of sporulation, the bacilli synthesize and secrete a wide variety of antibiotics and exoenzymes (10, 20). Many species in the genus *Bacillus* are widely used as sources of industrially important compounds. Bacilli possess a number of industrially valuable properties, including (i) the ability to excrete several different hydrolytic enzymes into the culture medium, (ii) the ability to produce antibiotics, (iii) the ability to incapacitate certain species of insects, (iv) the lack of pathogenicity, by most species, for humans, and (v) the ability to grow in simple culture media (for review, see reference 6).

This paper characterizes a member of the genus *Bacillus*, strain DSM704, as *Bacillus subtilis* and the production of 1-deoxynojirimycin (DN) by this strain. DN is a potent inhibitor of mammalian intestinal oligo- and disaccharidases (21) and trehalases (17). This compound has also been described in the literature as SG-1 (17) and moranolin (14, 15). Chemically, it is 1,5-dideoxy-1,5 imino glucitol. DN can be produced by several *Bacilli* (7, 21) and *Streptomycetes* (14, 15, 17) strains. In addition, DN also can be isolated from the leaves of mulberry trees (16).

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* DSM704 was obtained from W. Frommer (Bayer AG, Wuppertal, Federal Republic of Germany). Strain YB886 (*metB5 trpC2 xin-1* SPβ sensitive) is a laboratory strain originally from *B. subtilis* 168. Strain YB1011 is a prototrophic derivative of strain YB886. All bacteriophages used are described in Table 2.

Growth conditions and media. For the production of DN from various carbon sources, cells were grown for at least 7 days in minimal salts (per liter: K_2HPO_4, 14 g; KH_2PO_4, 6 g; $MgSO_4 \cdot 7H_2O$, 200 mg; $(NH_4)_2SO_4$, 2 g; $MnSO_4$, 1.7 mg; $Fe_2(SO_4)_3$, 28 mg; $ZnCl_2$, 7 mg; $CaCl_2$, 150 mg; plus 3% [wt/vol] carbon source). Incubation was in a rotary shaker (G-25; New Brunswick Scientific Co.) at 37°C and 250 rpm. Cells were grown in S medium (per liter: NZamine [Bayer AG], 3 g; yeast extract, 7 g; $CaCO_3$, 30 g; K_2HPO_4, 3 g; sorbitol, 70 g; adjusted to pH 6.8 with K_2CO_3) to measure the time course production of DN.

Transformation and transduction. The procedure for the isolation of DNA was described by Young and Spizizen (26). The development of competence and transformation procedure were by the method of Boylan et al. (2). For the selection of antibiotic resistance, a phenotypic expression time of 90 min was allowed. All transformations were done at saturating DNA concentrations (20 μg/ml). Transduction experiments and the preparation of bacteriophage stocks were as described by Yasbin et al. (25). *B. subtilis* YB886, unless otherwise specified, was the host for bacteriophage production.

Enzyme assays. β-Glucosidase activity was monitored by assaying the amount of *o*-nitrophenol released from *o*-nitrophenol-β-D-glucopyranoside by measuring absorbance at 420 nm on a Beckman DU-8 spectophotometer. Inhibition of β-glucosidase (almond extract; Sigma Chemical Co.) by culture supernatants was determined as follows: 0.05 mg of β-glucosidase (5 U/mg in 0.05 M sodium citrate buffer [pH 4.6]) per ml was mixed with 25 μl of culture supernatant and 30 μl of 0.05 M sodium citrate buffer (pH 4.6), and the reaction was started by the addition of 50 μl of *o*-nitrophenol-β-D-glucopyranoside (45 mg/ml), incubated for 5 min at 37°C, and terminated by the addition of 700 μl of $CaCO_3$ (0.2 M). The amount of *o*-nitrophenol released was quantified by measuring absorbance at 420 nm before and after the addition of *o*-nitrophenol-β-D-glucopyranoside.

Porcine sucrase was supplied by Bayer AG. Sucrase activity was monitored by assaying the amount of glucose released from sucrose. Glucose was detected by a modification of the method of Cawley et al. (3) as described below. Inhibition of sucrase by culture supernatants was determined as follows: 100 μl of 0.1 M sodium maleate buffer (pH 6.0) was mixed with 25 μl of culture supernatant and 25 μl of porcine sucrase (1 mg/ml; 0.3 U/mg in 0.1 M sodium maleate buffer [pH 6.0]), and the reaction was initiated by the addition of 100 μl of sucrose (20 mg/ml), incubated at 37°C for 60 min, and terminated by heating at 80°C for 2 min. Distilled water (800 μl) was added to each sample, and the amount of glucose liberated was determined as follows: 30 μl of PGO (horseradish peroxidase; 2.9 mg/ml; 520 U/mg)–glucose oxidase (0.133 mg/ml; 500 U/ml)–*O*-dianisidine (6 mg/ml) in 0.1 M sodium phosphate buffer (pH 6.0) was added and incubated for 10 min at 37°C, and the reaction was terminated by the addition of 1 ml of 50% H_2SO_4. The amount of glucose released was quantified by measuring the absorbance at 530

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nm before and after the addition of glucose. One sucrase inhibitory unit is defined as the amount of compound required to inhibit 2 U of sucrase activity by 50%.

Amylase was assayed by the method of Bernfield (1).

Heat resistance of DSM704 was determined by measuring the ability of cells to grow after incubation at 80°C for 20 min. Octanol resistance was determined by measuring the ability of cells to grow after incubation with 0.1% octanol for 10 min at 37°C.

RESULTS

Transformation of strain YB886. The ability of *B. subtilis* 168 derivatives to be transformed with DNA from other members of the genus has been well characterized (5, 22). In both studies referenced, a hierarchy of transformability was found, such that species which were more closely related evolutionarily to the laboratory strain were able to serve better as donors in transforming derivatives of *B. subtilis* 168. The ability of DNA from an erythromycin-resistant, rifampin-resistant derivative of strain DSM704 to transform laboratory strain YB886 for either antibiotic resistance or the auxotrophic requirements methionine and tryptophan was examined. Strain DSM704 chromosomal DNA was capable of transforming the laboratory strain for each of the markers tested, although at a lower efficiency as compared with that the homologous DNA transformation control (Table 1).

Infection of DSM704 with representative *Bacillus* bacteriophage. Various *Bacillus* species have been characterized by their ability to serve as hosts for one or more of the *Bacillus* bacteriophages. The ability of representative bacteriophages to form plaques on strain DSM704 was examined (Table 2). Most of the *Bacillus* bacteriophage groups, as described by Hemphill and Whitely (11), were examined, and none were able to give productive infections on strain DSM704. However, at least one bacteriophage, SPO2, has been shown to absorb to strain DSM704 (R. Marrero, personal communication). The inability of any of the bacteriophages tested to replicate in strain DSM704 precludes the use of this approach in characterization of the strain.

Production of DN from various carbon sources. The molecular structure of various carbon sources has been shown to influence the levels of proteases and bacitracin produced by cultures of *Bacillus licheniformis* (8, 9) and the amount of valine produced by a *B. subtilis* strain (4). Therefore, the ability of strain DSM704 to produce DN from a variety of carbon sources was analyzed. When DSM704 was grown on substrates metabolized via the tricarboxylic acid cycle as a sole carbon source, very low levels of DN were detected (Table 3). However, when strain DSM704 was grown on substrates metabolized via glycolysis, large quantities of DN were produced. The amount of DN produced from each of these compounds was monitored daily, for at least 7 days. The growth rate differed, depending on the nature of the carbon source. The amount of DN produced per milliliter of culture supernatant was the maximum amount of DN produced by that culture, irrespective of the length of time required to produce that amount; i.e., the maximum amount produced by bacteria grown on sorbitol occurred after 4 days of incubation, whereas the maximum amount achieved in cells grown on citrate took about 10 days.

Kinetics of DN production. The production of various metabolites over time by strain DSM704 was compared with the physiological state of the cells. Strain DSM704 proceeded through sporulation in a manner similar to that of other bacilli that have been studied (Fig. 1). If T = 0 was defined as the time that the cells end their exponential stage of growth, octanol-resistant cells were first detected between T = 8 and T = 9 (8 or 9 h after T = 0). The formation of heat-resistant spores followed in about 3 h. Members of the genus *Bacillus*

TABLE 1. Transformation of strain YB886[a]

Donor DNA	No. of transformants for the following marker[b]			
	Rif	Ery	*metB5*	*trpC2*
YB1011 (homologous)	220,000	93,000	42,000	360,000
DSM704 (heterologous)	9,500	22,000	930	1,200

[a] Transformation is the number of transformants that arise after the addition of 5 μg of DNA per 10^8 viable cells.

[b] The markers are: Rif, resistance to 15 μg of rifampicin per ml; Ery, resistance to 3 μg of erythromycin per ml; *metB5*, requires methionine for growth; and *trpC2*, requires tryptophan for growth.

TABLE 2. Phages tested

Phage group[a]	Phages
Virulent group:	
1	SP826, ϕ25
2	SP50
3	β22
4	NT[b]
5	SPP1
6	ϕ29
7	NT
Temperate	SPO2, ϕ105, ϕ3, ϕ3T
Pseudotemperate group:	
1	SP10
2	PBS1
3	NT
Other unclassified	SPβ, ρ11, PBSX, APR1[c]

[a] Phage groups have been classified as described in Hemphill and Whiteley (11).

[b] NT, None tested.

[c] APR1 is a phage specific for *B. amyloliquefaciens* strains.

TABLE 3. Production of DN from various carbon sources[a]

Carbon source	DN produced (mg/ml of culture supernatant)[b]
Fructose	1
Glucose	1
Sorbitol	1
Sucrose	1
Lactic acid	0.1
Glycerol	0.02
Pyruvate	0.02
Ribose	0.02
Succinate	0.002
Citrate	0.001
Malate	0.001

[a] Production of DN from medium containing 3% carbon source in a minimal salts solution is described in the text.

[b] Amount of DN produced was determined by a sucrose inhibitor assay as described in the text.

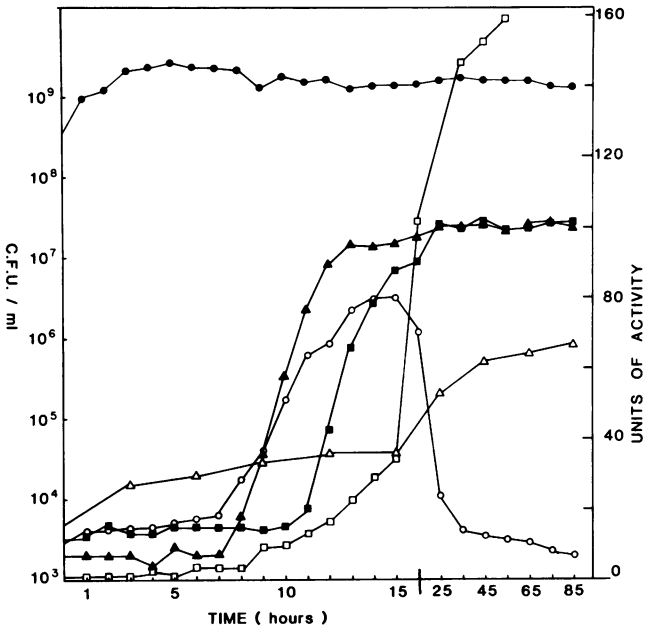

FIG. 1. Time course for the production of DN. The production of DN from a sorbitol-NZamine-yeast extract-based medium was monitored over several days. Growth was monitored colorimetrically (●) and remained relatively constant at ca. 300 Klett units (Klett Summerson Colorimeter with filter no. 66). The data indicate that octanol resistance (▲) occurs very quickly at the end of logarithmic growth. Production of a β-glucosidase inhibitor (○) occurs in the early idiophase period. This compound can be detected well before the production of heat-resistant spores (■). The production of DN (□) begins at the same time that heat-resistant spores are detectable, but the production phase lasts much longer. Production of amylase (△) occurs throughout the experimental period. All bacterial count determinations and colorimetric measurements are plotted on the logarithmic scale. All enzymatic determinations (DN production, β-glucosidase inhibitor, and amylase production) are plotted on the linear scale. Units are defined in the text. Viability is defined by the number of CFU per milliliter.

generally produce amylase concomitant with the process of sporulation (12). The data indicated that amylase production began at or near T = 8 for strain DSM704. Production of a β-glucosidase inhibitor in significant quantities did not begin until the appearance of octanol-resistant spores. Also, DN production did not begin until the spores had become heat resistant. However, production of DN continued even after the cells had reached their maximal sporulation level (Fig. 1). Whether DN production was mediated by the spore, the mother cell, or the nonsporulating component of the culture was unclear from the above experiment because only about 1% of the total population became heat resistant.

Utilization of DN. DN is a relatively simple compound and thus might serve as a reserve food storage material. Therefore, the ability of DN to be metabolized as a sole carbon or nitrogen source was examined. DN was not metabolized as a sole carbon or nitrogen source (Fig. 2). In addition, DN did not affect the growth rate of the cells (Fig. 2) grown in its presence. DN also had no effect on the time required for germination of spores (data not shown).

DISCUSSION

The relationship between the highly competent laboratory strains of *B. subtilis* 168 and additional industrially important species of this genus have been examined by two mechanisms: (i) the ability of DNA from each of these respective species to transform the laboratory strains of *B. subtilis* 168 (5, 24) and (ii) the ability of each strain to serve as a host for a series of bacteriophages (for review, see reference 11). Strain DSM704 was examined by the above criteria, and the data indicated that it was related to our laboratory strain of *B. subtilis* by the transformation assay. For instance, by using DNA-mediated transformation, it was found that the antibiotic resistance markers for erythromycin and rifampicin were transformed into our laboratory strain YB886 at an efficiency that ranged from 4 to 25% of the homologous control. These values indicated that the strain was more closely related to our laboratory strain than were *Bacillus amyloliquefaciens* H, *Bacillus pumilus*, or *B. licheniformis* strains that had been examined by Wilson and Young (22). However, by the same analogy, strain DSM704 was not as closely related to our laboratory strain as was *B. subtilis* W23 (see the values of Chilton and McCarthy [5]).

The bacteriophages capable of infecting *B. subtilis* encompasses the entire spectrum of virulence: lytic, temperate, pseudotemperate, and defective bacteriophages. Hemphill and Whitely (11) have reviewed the *B. subtilis* bacteriophages and classified them into several groups based on a wide variety of physical and biological properties. We tested representative samples of most of the viral groups depicted in their scheme, as well as several other unclassified bacteriophages, and found that none of the bacteriophages tested were capable of producing a viable infection on strain DSM704. The inability of bacteriophages to grow on this strain indicated that this strain was not related to the laboratory strain, but this result was in direct conflict with the data previously described in the transformation experiments. This paradox may be due to some undescribed control system that prevents the growth of any bacterio-

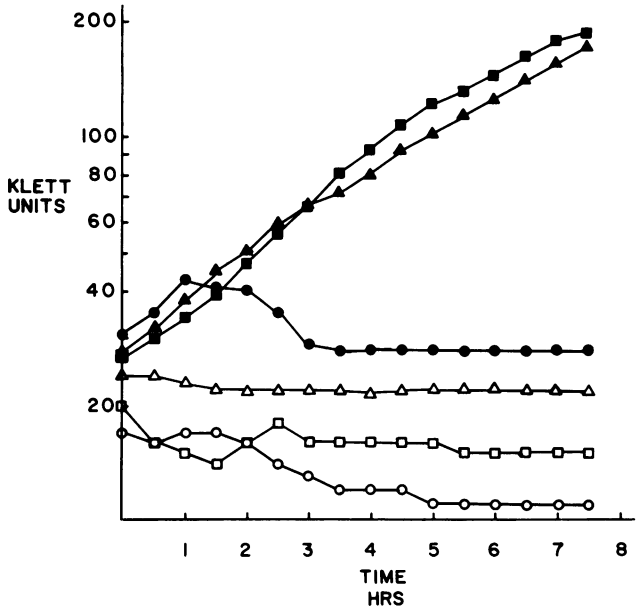

FIG. 2. Utilization of DN as a carbon or nitrogen source. Symbols: ○, buffer only (100 mM potasium phosphate, 1 mM $MgSO_4$ [pH 6.8]); △, buffer + 100 mM DN; □, 25 mM DN–2% $(NH_4)_2SO_4$; ●, 25 mM DN–1% glucose; ▲, 25 mM DN–1% glucose–2% $(NH_4)_2SO_4$; ■, 1% glucose–2% $(NH_4)_2SO_4$.

phage in this strain. For example, strain DSM704 may possess a restriction system, or it may possess modifications in its cell wall (23). The first conclusion seems most plausible since *B. subtilis* bacteriophage SPO2 can absorb to and inject its DNA but still not form plaques on this strain (R. Marrero, personal communication). Previously, bacteriophage interference systems have been characterized among several *Bacillus* strains (24). From the data generated by the sensitive transformation assay described above and from biochemical data (W. Frommer, personal communication), we have concluded that strain DSM704 is a *B. subtilis*.

Bacillus species are capable of undergoing differentiation at the end of exponential growth. The formation of heat-resistant spores in a culture of *B. subtilis* is intitiated when the culture enters stationary phase (10). Ohné and Rutberg (19) reported that growth on tricarboxylic acid cycle intermediates reduced the level of extracellular serine and metalloproteases without affecting the sporulation process. Leighton (13) suggested that this difference may be due to a delay in the onset of sporulation and that the reduction seen by Ohné and Rutberg (19) was only transient. The production of DN from various carbon sources was measured (Table 3). The results demonstrated that a decrease in production of DN occurred when cells were grown on tricarboxylic acid intermediates even though all cultures seemed to sporulate to the same extent. This observation would tend to support the results of Ohné and Rutberg (19). To further understand the production of DN, the kinetics of production were measured. The data in Fig. 1 indicate that DN was not produced in appreciable amounts until after the appearance of heat-resistant spores. This observation may explain the apparent discrepancy between Leighton (13) and Ohné and Rutberg (19). If the fermentations were carried out for a long enough period of time, significant levels of DN might be produced when the cells are grown on tricarboxylic acid cycle intermediates; however, this was not seen (unpublished data).

Since DN is a relatively simple compound, it should be possible to elucidate the intermediates in its biosynthesis from sorbitol. Since DN is a reduction product of nojirimycin, nojirimycin might serve as a precursor in DN biosynthesis. Nojirimycin can be detected in culture supernatants because it is a potent inhibitor of β-glucosidase (18). The data in Fig. 1 indicate that β-glucosidase inhibitory activity could be detected in the culture medium at about the same time as the appearance of octanol resistance. The quantity of this inhibitor rose to a steady state for several hours and then decreased. This decrease in β-glucosidase activity was paralleled by an increase in the inhibition of porcine sucrase activity. When the experiments were performed in a crude production medium, in which the yields of DN were much higher, there was an increase in the β-glucosidase inhibitory activity after the initial decrease. This increase was due to the fact that DN in large quantities will inhibit β-glucosidase (data not shown).

The large production of DN in nongrowing cells might be analogous to the uncontrolled or derepressed synthesis of primary metabolites such as vitamins or amino acids. The data in Fig. 2 indicate that the compound was not utilized by the cell as a sole energy or nitrogen source. Additional data indicated that DN had no effect on growing cells or on germinating spores (unpublished data).

In conclusion, we have characterized strain DSM704 as being a *B. subtilis* on the basis of biochemical and genetic data. We have demonstrated that DN was produced and acts as a classic secondary metabolite. Work is continuing in establishing the role of nojirimycin in the production of DN and the regulation of secondary metabolism.

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