Properties of Some Norvaline-resistant Mutants of *Bacillus subtilis*

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SUMMARY

DL-Norvaline inhibits growth of wild-type *Bacillus subtilis*. A number of mutants resistant to growth inhibition by this analogue were isolated and studied. Cross-feeding experiments and paper chromatography of culture supernatants indicated that the mutants excreted leucine and possibly valine and glutamate. Enzymic analysis indicated that the mutants were derepressed for acetohydroxy-acid synthetase and α -isopropylmalate synthetase; however, no derepression of threonine deaminase, dihydroxyacid dehydrase or transaminase B was observed.

INTRODUCTION

An important aid in the study of the control of biosynthetic pathways in micro-organisms has been the ability to isolate mutants having defective regulatory mechanisms. Such strains can often be obtained by selecting for mutants resistant to growth inhibition by analogues of pathway end-products. Several analogues have been successfully used to obtain regulatory mutants of the isoleucine, valine and leucine biosynthetic pathways. Calvo, Freundlich & Umbarger (1969) isolated a number of *Salmonella typhimurium* mutants resistant to the analogue 5',5',5'-trifluoroleucine. One class of mutants excreted leucine and possessed an α -isopropylmalate synthetase insensitive to inhibition by leucine. A second class of mutants excreted leucine and had derepressed levels of the leucine biosynthetic enzymes. The mutation mapped to one side of the leucine operon. A third class of mutants excreted isoleucine, valine and leucine and exhibited elevated levels of all of the branched-chain amino acid biosynthetic enzymes. Several mutants of *Escherichia coli* resistant to 4-thioisoleucine were obtained by Szentirmai, Szentirmai & Umbarger (1968). These strains had derepressed levels of threonine deaminase, dihydroxyacid dehydrase and transaminase B, but not of acetohydroxyacid synthetase or the isomeroreductase. These mutants appeared to possess an altered isoleucyl tRNA synthetase.

We describe strains of *Bacillus subtilis* resistant to DL-norvaline which show alterations in the regulation of several branched-chain amino acid biosynthetic enzymes.

METHODS

Strains. The wild-type strain of *Bacillus subtilis* used was WB746 (Chapman, 1972). The various derivatives of this strain were: NV20, NV26, NV35 and NV45 – resistant to growth inhibition by 200 μ g DL-norvaline/ml; SB271 – requires leucine; SB377R – requires isoleucine and valine; SB7 – requires isoleucine.

Growth. Liquid cultures were grown in 250 ml of medium as described by Chapman

(1972) and Chapman & Hull (1974). The cells were harvested by centrifugation, washed once with 0.05 M-potassium phosphate pH 7.0 + 0.5 mM-MgCl₂ + 0.01 mM-MnCl₂, and stored frozen until use. Cells to be used for the assay of α -isopropylmalate synthetase were washed with 0.05 M-tris pH 7.4 + 1.0 mM- α -ketoisovalerate + 0.1 mM-MnCl₂ + 20 % (v/v) glycerol.

Preparation of crude extracts. This was usually done by lysing the cells in a buffer containing 1.0 mg lysozyme and 10 μ g DNAase/ml for 30 min at 37 °C (for threonine deaminase assays, cells were lysed for 60 min at room temperature). Cell debris was removed by centrifugation at 27000 g for 30 min at 0 to 5 °C (for threonine deaminase, centrifugation was at room temperature). For acetohydroxyacid synthetase, the lysis buffer contained 0.05 M-potassium phosphate pH 7.0 + 0.05 M-KCl + 5 mM-MgCl₂ + 80 μ g thiamine pyrophosphate (TPP)/ml + 10 μ g FAD/ml. For threonine deaminase and transaminase B the buffer was 0.05 M-potassium phosphate pH 7.0 + 0.1 mM-pyridoxal-5-phosphate + 2 mM-MgCl₂. For dihydroxyacid dehydrase the buffer was the same except pyridoxal phosphate was omitted. For α -isopropylmalate synthetase the lysis buffer was the same as the wash buffer described above. Before use, the crude extract was filtered through a column of Sephadex G-25 at 0 to 5 °C and equilibrated with the same buffer without α -ketoisovalerate.

Enzyme assays. Acetohydroxyacid synthetase was assayed by the method of Bauerle *et al.* (1964) and acetoin determined by the method of Westerfield (1945). The reaction mixture contained (per 1.0 ml): 100 μ mol potassium phosphate pH 8.0; 20 μ mol pyruvate; 10 μ mol MgCl₂; 80 μ g TPP; 20 μ g FAD. For controls, enzyme was added after termination of the reaction with H₂SO₄. Threonine deaminase was assayed by the method of Hatfield & Umbarger (1971). The reaction mixture contained (per 1.0 ml): 100 μ mol potassium phosphate pH 8.0; 40 μ mol L-threonine; 0.1 μ mol pyridoxal-5-phosphate. For controls, enzyme was added after termination of the reaction. Dihydroxyacid dehydrase was assayed as described by Kiritani & Wagner (1970). The reaction mixture contained (per 0.5 ml): 50 μ mol potassium phosphate pH 8.0; 15 μ mol dihydroxyisovalerate; 4 μ mol MgCl₂. For controls, enzyme was added after termination of the reaction. Transaminase B was assayed by the method of Duggan and Wechsler (1973). The reaction mixture contained (per 1.0 ml): 100 μ mol potassium phosphate pH 8.0; 25 μ mol L-valine; 20 μ mol α -ketoglutarate; 0.1 μ mol pyridoxal-5-phosphate. Valine was omitted from the controls. α -Isopropylmalate synthetase was assayed using the DTNB procedure of Calvo, Bartholomew & Stieglitz (1969). The reaction mixture contained (per 0.5 ml): 50 μ mol tris pH 8.0; 50 μ mol KCl; 2.5 μ mol α -ketoisovalerate; 1.0 μ mol acetyl CoA; 0.2 μ mol MnCl₂. α -Ketoisovalerate was omitted from the controls. Activity of all assays was linear with enzyme concentration under the conditions used. Specific activities of all enzymes are expressed as nmol product formed/min/mg protein. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin used as a standard.

Isolation of resistant mutants. Analogue-resistant mutants were isolated as described by Chapman & Hull (1974) except that the cells were spread on to minimal medium plates containing 200 μ g DL-norvaline/ml.

Paper chromatography of cultural supernatants. Cells were initially grown and harvested as described by Chapman & Hull (1974). They were washed once with a special minimal medium and used to inoculate 200 ml of special minimal medium in a 400 ml beaker to give an E_{660} of 0.67. The special minimal medium contained (per litre distilled water): 1.0 g (NH₄)₂HPO₄, 0.5 g KH₂PO₄, 0.25 g (NH₄)₂SO₄, 0.5 % glucose, and 0.1 g MgCl₂. The cells were grown with vigorous stirring at 37 °C until the extinction reached 2.0 (2½ to 3½ h). The pH of the culture was maintained between 6.6 and 7.0 by adding NH₄OH. The

cells were removed from the liquid by centrifugation. Twenty-five ml of the supernatant was desalted using a column of Dowex 50W by the procedure of Mueller, Bowman & Herranen (1955). The eluate containing the amino acids was evaporated to dryness, and the residue resuspended in 1.6 ml of 10% (v/v) isopropanol.

Paper chromatography was carried out on 22 × 26 cm sheets of Whatman No. 1 chromatography paper. The solvents used were butanol–acetic acid–water (60:15:25, by vol.), phenol–water (8:2, w/v), ethanol–water–NH₄OH (90:5:5, by vol.), and *t*-butanol–water–ethylmethylketone–diethylamine (40:40:20:4, by vol.). Amino acids were located by dipping the dried papers into a solution of 0.2% ninhydrin in acetone. Colours were allowed to develop for at least 9 h at room temperature.

Transformation. Transformation procedures were carried out as described by Chapman & Hull (1974).

Chemicals. TPP, FAD, α -ketoglutarate, α -ketoisovalerate, acetyl CoA and bovine serum albumin were from Sigma, potassium pyruvate from Calbiochem, DL-norvaline from Nutritional Biochemicals, and sodium α,β -dihydroxyisovalerate was the gift of Dr H. E. Umbarger, Purdue University, U.S.A.

RESULTS

Isolation of resistant mutants

In an effort to obtain mutants of *B. subtilis* derepressed for acetohydroxyacid synthetase, a large number of branched-chain amino acid analogues were screened for their ability to inhibit growth of wild-type cells. Of these, DL-norvaline proved inhibitory. Using a concentration of 200 μ g/ml, 50 mutants resistant to growth inhibition were then isolated. All were assayed for acetohydroxyacid synthetase, and 20 were derepressed. Four independent isolates (NV20, NV26, NV35 and NV45) showing different degrees of derepression were selected for further study.

Excretion of amino acids by the mutants

During isolation of the mutants it was noticed that some of the resistant colonies appearing on analogue-containing plates were surrounded by zones of background growth. This phenomenon often indicates that the mutant is overproducing and excreting a compound which can reverse the inhibitory action of the analogue, allowing growth of sensitive cells. In order to test this hypothesis, the wild-type strain and each NV strain were streaked first parallel to and then away from each other on analogue-containing plates. Growth of the wild-type strain occurred only where it had been streaked adjacent to the mutant strain. All four NV mutants were able to promote the growth of the wild-type strain, although NV20 was less able than the other three strains. These results, therefore, suggest that the resistant mutants excrete a compound or compounds which can reverse the inhibitory effect of norvaline.

The initial approach to identifying the excreted compound(s) was to perform cross-feeding experiments. The resistant mutants were tested for their ability to promote the growth of strains SB7 (Ile⁻), SB377R (Ile⁻, Val⁻) and SB271 (Leu⁻) when the resistant and auxotrophic strains were streaked parallel to each other on minimal medium plates. With strain SB377R the experiments were also done using minimal medium + isoleucine and minimal medium + valine. As controls, the auxotrophs were streaked adjacent to the wild-type strain. The wild-type strain did not feed any of the auxotrophs. With the NV mutants only slight cross-feeding was observed with strains SB7, SB377R on minimal medium, and SB377R on minimal medium + valine. However, considerable growth of strains SB271 and

Fig. 1. Tracing of a paper chromatogram of the cultural supernatants of strains WB746 and NV45 along with several amino acid standards. The solvent system used was phenol-water. Concentrates of cultural supernatants were prepared as described in Methods. Striped circles indicate intense ninhydrin-positive spots, and open circles indicate faint ninhydrin-positive spots.

SB377R on minimal medium+isoleucine occurred when they were streaked next to the resistant mutants. The cross-feeding characteristics of all the NV mutants were similar, which suggests that the mutants may excrete leucine and valine.

The second approach used to identify excreted amino acids was paper chromatography of culture supernatants. Figure 1 presents a tracing of a chromatogram of the supernatants of NV45, WB746 and several amino acid standards run in phenol-water. Mutant NV45 showed an intense ninhydrin-positive spot having an R_F value of 33, whereas WB746 showed only a weak spot at this point. These spots co-chromatographed with glutamate. Both strains showed weak spots with an R_F of 52 (co-chromatographing with alanine). Weak spots with R_F values of 73 to 74, which ran with valine, were also observed. However, NV45 showed an intense spot having an R_F value of 80 which was not shown by strain WB746. This spot chromatographed with leucine. Results consistent with these were also obtained with the solvents butanol-acetic acid-water, ethanol-water- NH_4OH , and *t*-butanol-water-ethylmethylketone-diethylamine. Qualitatively, all of the mutants gave

Table 1. *Enzyme levels in the norvaline-resistant mutants*

Growth of cells, preparation of extracts and assay of enzymes were described in Methods.

Strain	Medium	Specific activity/nmol product formed/min/mg protein				
		Thr D	AHAS	DHAD	Trans B	IPMS
WB746	M	141.0	35.5	19.6	35.2	11.4
NV20	M	123.8	66.9	16.1	21.3	37.0
NV26	M	119.6	160.5	20.2	22.3	86.5
NV35	M	106.5	181.8	20.0	21.3	87.8
NV45	M	94.4	223.5	18.6	19.9	85.8
WB746	M+IVL	70.3	5.7	7.2	11.5	1.0
NV20	M+IVL	79.9	31.6	3.1	12.8	15.6
NV26	M+IVL	84.1	110.9	2.5	13.6	52.6
NV35	M+IVL	72.0	132.3	3.0	13.6	40.9
NV45	M+IVL	65.6	137.3	4.9	14.1	57.1

M, minimal medium; I, isoleucine (50 µg/ml); V, valine (50 µg/ml); L, leucine (40 µg/ml); Thr D, threonine deaminase; AHAS, acetohydroxyacid synthetase; DHAD, dihydroxyacid dehydrase; Trans B, transaminase B; IPMS, isopropylmalate synthetase.

similar results. Quantitatively, however, there were some variations among the four mutants as well as between different supernatants of the same mutant. Strain NV20 showed the weakest accumulation. Some supernatants showed only a weak spot corresponding to glutamate. However, with three supernatants of the wild-type strain, either no spot or only a very weak spot chromatographing with leucine was ever observed. The chromatographic experiments therefore support the results of the cross-feeding experiments, that the mutants excrete leucine and possibly valine.

Enzyme levels in the resistant mutants

The specific activities of a number of the branched-chain amino acid biosynthetic enzymes were determined and compared with those present in the wild-type strain (Table 1). When grown in minimal medium, NV20 was derepressed about two-fold for acetohydroxyacid synthetase, NV26 and NV35 about 4.5- to 5-fold, and NV45 about sixfold. When the strains were grown in minimal medium plus excess isoleucine, valine and leucine, some repression of enzyme synthesis occurred in all the mutants, but not to the extent that it occurred in WB746. On the other hand, threonine deaminase, dihydroxyacid dehydrase and transaminase B were not derepressed in any of the mutants. Supplementation of the medium with isoleucine, valine and leucine resulted in complete repression of these three enzymes. However, the first leucine enzyme, α -isopropylmalate synthetase, was derepressed in all of the mutants, NV20 about three-fold and the other strains about 7.5-fold. As observed with acetohydroxyacid synthetase, growth of the cells in the presence of isoleucine, valine and leucine resulted in only partial repression of enzyme synthesis. None of the mutants showed significant alterations in the extent of feedback inhibition of threonine deaminase by isoleucine, acetohydroxyacid synthetase by valine or of α -isopropylmalate synthetase by leucine.

Preliminary genetic mapping

Linkage of norvaline resistance to the leucine operon was tested by determining the cotransfer of mutant loci with the leucine marker of strain SB271 in a repulsion cross of the type *nov^rleu⁺* (donor) $\times$ *nov^rleu⁻* (recipient). The resistance markers in strains NV45 and NV20 showed 10 and 40 % cotransfer, respectively, with the reference marker at limiting DNA

concentrations. Thus, the gene conferring analogue resistance appears to be carried on the same molecule of transforming DNA as the genes specifying the leucine enzymes.

DISCUSSION

The four norvaline-resistant mutants examined in this study all appear to have similar properties. Only quantitative differences in the extent of derepression of acetohydroxyacid synthetase and α -isopropylmalate synthetase, and in the amounts of excreted amino acids, have so far been observed. It is therefore probable that the mutation in each strain involves the same gene, although more detailed genetic studies will be necessary to confirm this.

No mutants of *E. coli* or *S. typhimurium* resistant to norvaline have been reported. Of the analogue-resistant strains that have been studied, none show a pattern of derepression of the branched-chain amino acid biosynthetic enzymes similar to the norvaline mutants described here. However, Ward & Zahler (1973*a,b*) have isolated mutants of *B. subtilis* resistant to 4-azaleucine which are derepressed for the leucine enzymes and acetohydroxyacid synthetase. The mutation maps to one side of the leucine operon. Thus, our norvaline-resistant strains are phenotypically similar to their azaleucine-resistant mutants, and they may also be similar genetically.

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