JOURNAL OF BACTERIOLOGY, July 1972, p. 86-93

Vol. 111, No. 1
Printed in U.S.A.

Synthesis of Pulcherriminic Acid by *Bacillus subtilis*

ROBERT L. UFFEN[1] AND E. CANALE-PAROLA
Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01002

Received for publication 10 November 1971

The pathway of pulcherriminic acid synthesis in *Bacillus subtilis* strains AM and AM-L11 (a leucine-requiring auxotroph) was investigated. Determinations of radioactivity in pulcherriminic acid synthesized by cells growing in media containing ^{14}C-labeled amino acids indicated that *B. subtilis* produced pulcherriminic acid from L-leucine. The organism utilized the carbon skeletons of two L-leucine molecules to synthesize one molecule of pulcherriminic acid. Similar results were obtained with starved cell suspensions. Growing cells formed significant amounts of pulcherriminic acid only in media including a carbohydrate such as starch. However, carbohydrate carbon was not required for the synthesis of pulcherriminic acid molecules. Data obtained with cell suspensions supported the hypothesis that cyclo-L-leucyl-L-leucyl is an intermediate in pulcherriminic acid biosynthesis and indicated that molecular oxygen is required for the conversion of cyclo-L-leucyl-L-leucyl to pulcherriminic acid. A pathway for the synthesis of pulcherrimin from L-leucine in *B. subtilis* is proposed.

A number of bacteria and the yeast *Candida pulcherrima* produce the red pigment pulcherrimin when growing in media containing ferric salts (3-7, 9-11, 15-17). Studies with a pulcherrimin-producing strain of *Bacillus subtilis* (10) showed that the pigment is the product of a nonenzymatic reaction between iron present in the growth medium and pulcherriminic acid excreted by the cells (Fig. 1). Pulcherrimin was characterized as a ferric chelate or salt of pulcherriminic acid (2,5-diisobutyl-3,6-dihydroxypyrazine-1,4-dioxide or a tautomeric form of this compound), as briefly reviewed in a previous publication (10).

The physiological function of these cyclic molecules is not known. Kupfer, Uffen, and Canale-Parola (10) concluded that the nonenzymatic formation of pulcherrimin from pulcherriminic acid is a fortuitous event depending on the availability of iron in the environment, and has no readily apparent effect on cell metabolism. Thus, pulcherriminic acid, rather than pulcherrimin, may be considered the pyrazine compound of biological significance. The metabolic steps leading to the synthesis of pulcherriminic acid in *C. pulcherrima* were studied by MacDonald (12). He found that pulcherriminic acid was derived from L-leucine and that cyclo-L-leucyl-L-leucyl was an intermediate.

Growing cells of aerobic sporeforming bacteria produce pulcherrimin in media including amino acids and a carbohydrate such as starch (5, 10, 15, 16). It was observed that, when the carbohydrate was omitted from the medium, only a trace amount of pigment was formed, even though the final cell yield was not significantly affected. The requirement for a carbohydrate suggested the possibility that *Bacillus* species synthesized pulcherriminic acid via a metabolic route different from that present in *C. pulcherrima*. The investigation described in this report was undertaken to study the pathway of pulcherriminic acid synthesis in *B. subtilis*.

MATERIALS AND METHODS

Organisms, culture media, and growth conditions. *B. subtilis* strains AM (10) and AM-L11 were used in this studv. *B. subtilis* AM-L11, a pulcherrimin-forming leucine auxotroph, was obtained from the parent AM strain by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1).

The organisms were grown in medium P containing per 100 ml of distilled water: soluble starch (Difco), 2.0 g; yeast extract (Difco), 1.0 g; ferric ammonium citrate (brown, Allied Chemical and Dye Corp., New York, N.Y.), 0.01 g. The *p*H of this medium was adusted to 6.9 ± 0.1 with an aqueous solution of KOH before sterilization. In some experi-

[1] Present address: Department of Microbiology and Public Health, Michigan State University, East Lansing, Mich. 48823.

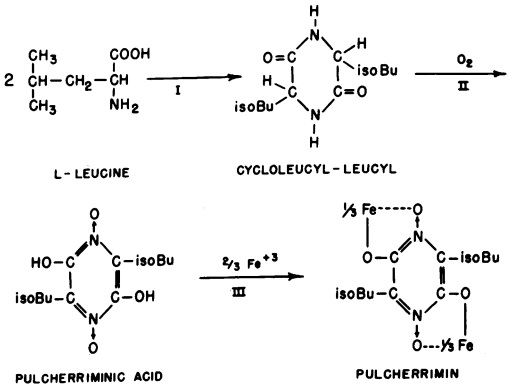

FIG. 1. *Proposed pathway of pulcherrimin synthesis from leucine in B. subtilis. Reaction I may involve more than one step. Reaction III is extracellular and does not involve the participation of an enzyme.*

ments, medium P was modified by the addition of separately sterilized potassium phosphate buffer, *p*H 6.9, to a final concentration of 0.1 M (medium PB). The *p*H of this medium, after sterilization, was 6.9. Medium CH, used in radioactive tracer studies, was identical to medium PB, except that 1 g of vitamin-free casein hydrolysate (Nutritional Biochemical Co.) was added per 100 ml, instead of the yeast extract. A chemically defined (CD) medium contained per 100 ml of distilled water: soluble starch, 1.0 g; L-glutamic acid, 0.4 g; biotin, 5×10^{-6} g; thiamine-hydrochloride, 5×10^{-5} g; ferric ammonium citrate and $MgSO_4 \cdot 7H_2O$, 10^{-2} g each; $MnSO_4 \cdot H_2O$, 5×10^{-3} g; NH_4Cl, 0.2 g. The *p*H was adjusted to 6.9 with an aqueous solution of KOH before sterilization. The medium was buffered with 0.25 and 0.1 g of K_2HPO_4 and KH_2PO_4, respectively, per 100 ml (sterilized separately from the other medium components). The final *p*H of the medium was 6.9.

The iron salt was added to all growth media to trap the excreted pulcherriminic acid as its insoluble iron chelate (pulcherrimin) since pulcherriminic acid is unstable in solution.

Cells were grown at 30 C in Erlenmeyer flasks filled to one-fifth capacity with the medium. The cultures were aerated on a New Brunswick Scientific Co. rotary shaker, model VS-100, at 200 rev/min.

Assay for L-leucine. Conventional microbiological techniques (13) were used to assay for L-leucine. Assay organisms were *Escherichia coli* 1177 (*leu*$^-$, *thr*$^-$, *vit* B_1^-), obtained from C. B. Thorne (Department of Microbiology, Univ. of Massachusetts, Amherst), and *Leuconostoc mesenteroides* P-60 (ATCC 8042), a leucine-requiring auxotroph. The latter organism was used to determine concentrations of L-leucine in CD medium (supplemented with this amino acid). Both assay organisms were used for the quantitative determination of L-leucine in radioactive tracer studies. *L. mesenteroides* P-60 was grown in the commercially prepared (Difco) assay medium of Steele et al. (14). *E. coli* 1177 was grown in the minimal salts medium described by Anderson and Wood (2) to which the following additions were made per 100 ml of medium: L-threonine, 0.1 g; thiamine-hydrochloride, 0.01 g; and glucose (sterilized separately), 1.0 g. Either chromatographically pure leucine (L or DL) or leucine-containing samples were added to both assay media. Growth was measured turbidimetrically. The amount of D-leucine added to CD medium was determined gravimetrically.

Measurements of pulcherriminic acid and pulcherrimin. With the exception of those studies in which radioactively labeled compounds were used, pulcherriminic acid or pulcherrimin was estimated by a method similar to that of Canale-Parola (5). Cell samples containing pulcherrimin were washed twice with methanol and once with distilled water before extraction with 2 M NaOH. The amount of pulcherriminic acid present as the sodium salt was determined spectrophotometrically at 410 nm using a standard absorption curve obtained with purified pulcherriminic acid (16) dissolved in 2 M NaOH. Although pulcherriminic acid dissolved in 2 M NaOH gradually breaks down when exposed to sunlight, ordinary laboratory illumination does not affect this assay [the absorbancy was measured 30 min after addition of the base (5)]. The assays were made at room temperature (22 to 23 C). Cycloleucyl-leucyl interfered with absorption measurements and, when present, was removed from the pigmented cell pellet by treatment with boiling 95% ethyl alcohol.

Purification of pulcherriminic acid. In experiments involving the use of radioactively labeled compounds, pulcherriminic acid was purified and estimated as follows. *B. subtilis* AM-L11 grown for 36 to 48 hr in 200 ml of medium CH was harvested, together with the pigment it produced, by centrifugation. The growth medium included the ^{14}C-labeled compound under test, as specified below and in Table 2. The pellet was washed three times with methanol and once with distilled water. Crude pulcherrimin (5 to 10 mg) was obtained by extracting the pellet with 2 M NaOH, removing the insoluble material by centrifugation, and reconstituting the pigment by addition of $FeCl_3$ and acid to the yellow supernatant liquid (5, 10). The crude pigment, suspended in 10 ml of 1.2 M HCl, was heated for 10 min at 100 C and recovered by centrifugation. This heating procedure was repeated two additional times. The acid-treated pulcherrimin was washed with distilled water and dissolved in 2 to 4 ml of 2 M NaOH. The resulting yellow solution, containing sodium pulcherriminate, was cooled to 4 C, and the precipitated ferric hydroxide was removed by centrifugation (20,000 $\times$ *g*, 10 min, 4 C). The clear yellow supernatant liquid was brought to a volume of 4 to 6 ml with cold (4 C) distilled water and was slowly acidified with cold 12.1 M HCl until a brown-red precipitate began to appear. Titration was continued to *p*H 5.0 to 3.5 with cold 1.2 M HCl. The brown-red precipitate was collected by centrifugation, washed twice with cold distilled water, and suspended in 0.4 to 0.7 ml of a mixture of 95% ethyl alcohol (redistilled), glass-distilled water, and M potassium phosphate buffer, *p*H 8.6 (5:5:1, v/v/v). The suspension was heated in warm water (50 to 60 C)

for 1 min to aid solubilization. After removal of the insoluble material by centrifugation (20,000 × *g*, 10 min, 4 C), the supernatant liquid was placed onto a Sephadex LH-20 (Pharmacia Fine Chemicals, Inc.) chromatographic column (28 by 2 cm) at 4 C. Pulcherriminic acid was eluted from the column with the alkaline ethanol-water-phosphate buffer solvent system, as previously described (16). The amounts of pulcherriminic acid eluted were determined spectrophotometrically at 410 nm with a standard curve prepared with purified pulcherriminic acid (16) dissolved in the alkaline ethanol-water-phosphate buffer solvent system.

Similar procedures were used for the purification and estimation of pulcherriminic acid from starved cell preparations (see Table 2, expt 5).

Measurements of radioactivity. Samples of pulcherriminic acid eluted from Sephadex LH-20 columns and samples of other ^{14}C-labeled compounds were dried on stainless-steel planchets, and their radioactivity was measured in a Nuclear Chicago model 181B decade scaler.

In some experiments (see Table 2, expt 5; Fig. 3), radioactivity was measured by scintillation techniques. Pulcherriminic acid samples eluted from Sephadex LH-20 columns were dried in vacuo and dissolved in 1 ml of 0.1 M NaOH-methanol solution. Dried residues containing ^{14}C-labeled amino acids were dissolved in 1 ml of hydroxide of hyamine (10X; Packard Instruments Co.). Each dissolved sample was added to scintillation fluid consisting of toluene, spectral grade, 100 ml; dimethyl POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene], 0.02 g; PPO (2,5-diphenyloxazole), 0.5 g. The total volume per scintillation vial was 15 ml. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer, model 3003.

Radioactive tracer studies with starved cells. One-liter Erlenmeyer flasks, each containing 200 ml of medium CH, were inoculated with *B. subtilis* AM-L11 (10^7 cells/flask) grown on medium P agar plates. The cells were harvested by centrifugation shortly before the beginning of pigment synthesis (after 33 hr of incubation under the growth conditions previously stated). Cells from 800 ml of CH medium cultures were suspended in 100 ml of phosphate-mineral buffer (0.1 M potassium phosphate buffer, *p*H 6.9, containing 0.01 g each of ferric ammonium citrate and $MgSO_4 \cdot 7H_2O$ per 100 ml). The suspension was incubated at 30 C for 4 to 4.5 hr with agitation (New Brunswick Scientific Co. rotary shaker, model VS-100, 200 rev/min). Finally the cells were collected by centrifugation, were washed with and suspended in phosphate-mineral buffer, and their level of pigmentation was determined (some pigment was formed during starvation).

Each reaction mixture contained, per 125-ml Erlenmeyer flask: L-leucine-*U*-^{14}C, 1,000 μmoles (1,000 counts per min per μmole); starved cells, 0.6×10^{11} to 1×10^{11}; and phosphate-mineral buffer to a final volume of 50 ml. Incubation was for 3 hr at 30 C on the rotary shaker (200 rev/min). Reaction mixtures incubated under the same conditions, but in the absence of leucine, produced low levels of pigment which were assayed to determine pigment synthesis due to endogenous metabolism. Under the conditions used, significant cell lysis did not occur, as determined by direct cell counts.

Pulcherriminic acid synthesis by cell suspensions. The role of cycloleucyl-leucyl and L-leucine in pulcherriminic acid synthesis was investigated by the following techniques. *B. subtilis* AM-L11 was grown in medium CH until pigment began to be formed, as determined visually (34 to 36 hr of growth). The cells were harvested by centrifugation, washed three times in 0.1 M potassium phosphate buffer, *p*H 6.9, and finally suspended in the same buffer.

Reaction mixtures in Warburg vessels included, per flask: L-leucine (30 μmoles) or cycloleucyl-leucyl (17.1 μmoles); washed cells, 3×10^{10} to 5×10^{10}; phosphate-mineral buffer, to a final volume of 3.2 ml. Incubation was at 30 C in air or N_2 (see Table 3), at 60 oscillations per min (GME Lardy circular Warburg). Cycloleucyl-leucyl was not completely soluble, and the undissolved portion adhered to the walls of the reaction vessels; it was periodically resuspended in the reaction liquid during incubation. The number of cells in the reaction mixtures remained approximately constant throughout the experiments, as determined by direct microscope cell counts.

Other procedures. Cycloleucyl-leucyl isomers were prepared by a modification of a procedure described by E. E. Fisher and M. T. Tetenbaum [U.S. patent no. 3,121,717 (Cl. 260-268) to A. E. Staley Manufacturing Co., Decatur, Ill., 1964]. Cyclo-D-leucyl-L-leucyl was synthesized and purified as described by MacDonald (12). The melting point of the cycloleucyl-leucyl preparations was in agreement with that published by Goto (8). Free leucine was not detected in the cycloleucyl-leucyl preparations either by thin-layer chromatography on Silica Gel G plates (5) or by infrared spectroscopy (Perkin-Elmer grating infrared spectrophotometer, model 257; solid in KBr).

A Petroff-Hausser bacterial counter was used to determine cell numbers.

Chemicals. The following chemicals were obtained from the suppliers indicated in parentheses: nonlabeled amino acids (Nutritional Biochemicals Corp., Cleveland, Ohio, or Calbiochem, Los Angeles, Calif.); L-leucine-*U*-^{14}C, L-tyrosine-*U*-^{14}C, and L-aspartic acid-*U*-^{14}C (Schwarz Bio-Research Inc., Orangeburg, N.Y.); L-leucine-*1*-^{14}C and DL-leucine-*2*-^{14}C (Calbiochem); L-alanine-*U*-^{14}C and L-glutamic acid-*U*-^{14}C (Intern. Chem. and Nuclear Corp., City of Industry, Calif.); starch-*U*-^{14}C (Nuclear Chicago Corp., Des Plaines, Ill.); *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.); and dimethyl POPOP and PPO (Packard Instrument Co., Downer's Grove, Ill.).

RESULTS

Conditions affecting pigment production by growing cells. *B. subtilis* growing in medium P initiated pigment synthesis only after the starch in the medium was almost depleted. Depending on the growth conditions, pulcher-

rimin formation was first detected in the cultures 12 hr or more after inoculation, at cell densities of 2×10^8 to 6×10^8 cells/ml. When all starch had been utilized, the *p*H of the cultures had decreased to 5.9 ± 0.1 (the *p*H was 6.9 immediately after inoculation). However, as growth continued and other medium components (probably amino acids) were being metabolized, the *p*H increased to reach a final value of 7.5 ± 0.1.

To determine whether a given concentration of hydrogen ions in the medium was needed to initiate pigment synthesis, the amounts of pigment present and the cell concentrations were measured during growth at constant *p*H values (Fig. 2). The results indicated that the decrease in *p*H during starch metabolism was not a requirement for pigment synthesis initiation.

As previously mentioned, omission of carbohydrate from the growth medium resulted in a drastic decrease in the amount of pigment formed. This suggested that growing cells required a carbohydrate or a product of carbohydrate metabolism for pigment synthesis. The *p*H of cultures to which carbohydrate was not added (medium P or PB without starch) did not decrease, but increased directly from the initial value of 6.9 to approximately 7.4. However, the increase in *p*H was not a determining factor (or not the only determining factor) in preventing pigment synthesis, since cells grown at constant *p*H values (6.5 and 6.9) yielded only traces of pigment in the above-mentioned starch-free media. Addition of starch to this type of culture, in the late exponential or stationary phase of growth, resulted in greatly increased pigment formation during continued incubation.

CD medium, containing glutamic acid as the only amino acid, supported pigment production by growing cells of *B. subtilis* AM. A typical final yield of pigment in this medium was 1 mg of pulcherrimin per 1.2×10^{11} cells. Addition of L-leucine, L-arginine, and L-aspartic acid to this medium, either singly or in combinations, increased (up to doubling) the final pigment yield relative to cell number. Other amino acids did not stimulate pigment production significantly. Addition of L-tryptophan (50 μg/ml) to CD medium totally inhibited pigment synthesis, in contrast to the reported stimulatory effect of this compound on pulcherrimin synthesis by growing cells of *Micrococcus violagabriellae* (4).

When *B. subtilis* AM was grown in CD medium supplemented with different amounts of L-leucine, the initiation of pigment synthesis was delayed in response to increased amounts of added amino acid (Table 1). Although the final amount of pigment accumulated in the medium by *B. subtilis* AM did not account for all of the L-leucine utilized during growth (as determined by microbiological assay), higher pigment yields were obtained by increasing the initial concentration of L-leucine in the medium (Table 1). It appeared that the presence of L-leucine in the growth medium had the twofold effect of delaying the initiation of pigment formation and of increasing the final pigment yield. The effect of the initial leucine

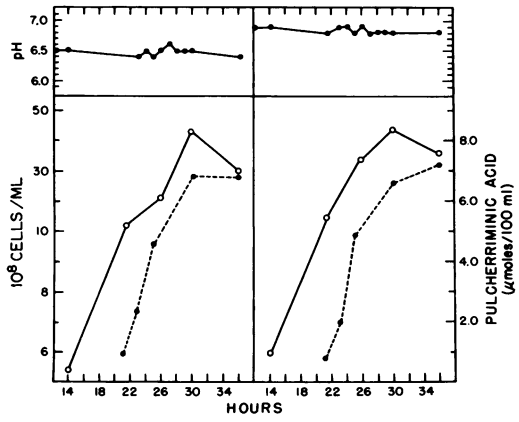

FIG. 2. *Synthesis of pigment by B. subtilis AM-L11 growing at constant pH in medium PB. The cultures were maintained at approximately pH 6.5 (left side of figure) and 6.9 (right side of figure). Pigment is expressed as pulcherriminic acid. Direct cell count* (O——O); *amount of pulcherriminic acid* (●----●); *pH* (●——●).

TABLE 1. *Effect of leucine on pulcherrimin synthesis by cells of Bacillus subtilis AM growing in a chemically defined (CD) medium*

Added to CD medium	Initial concn of amino acid (μmoles/ml)	Cell yield[a]	Delay in pulcherrimin synthesis (hr)	Pulcherrimin formed[b]
No addition		2.8×10^9	0[c]	80
L-Leucine	3.8	2.8×10^9	2	120
L-Leucine	11.4	2.7×10^9	6	150
L-Leucine	20.0	3.0×10^9	8	160
D-Leucine	7.6	2.7×10^9		0
D-Leucine	15.3	2.7×10^9		0

[a] Cells per milliliter. Direct cell count after 34 hr of incubation. Some cell lysis occurred when incubation was continued.

[b] Nanomoles per ml. Assayed after 36 hr of incubation.

[c] Pulcherrimin appeared after 16 hr of incubation.

concentration on the inception of pigment synthesis suggests that this amino acid plays a regulatory role in pulcherriminic acid formation. Addition of D-leucine to CD medium prevented pigment production (Table 1), an indication that, in *B. subtilis* AM, D-leucine may act as a competitive inhibitor of pulcherriminic acid synthesis from L-leucine.

Incorporation of L-leucine into pulcherriminic acid. The role of amino acids in pigment synthesis was investigated by measuring the radioactivity of pulcherriminic acid produced by *B. subtilis* AM-L11 in the presence of ^{14}C-labeled amino acids. To determine the radioactivity present in pulcherriminic acid synthesized by the cells, it was essential to remove contaminating substances from pulcherriminic acid preparations as described above. Purification of pulcherriminic acid from these crude preparations was accomplished with Sephadex LH-20 chromatographic columns (see above and reference 16). When crude preparations of pulcherriminic acid synthesized by *B. subtilis* grown in the presence of L-leucine-*U*-^{14}C were resolved by gel filtration through these columns, essentially all the eluted ^{14}C label coincided with visible light-absorbing bands (Fig. 3). The material in band 2 (Fig. 3), which was yellow and exhibited maximal visible light absorption at 410 nm, consisted of pulcherriminic acid (16). Material from this band was used to measure the radioactivity of pulcherriminic acid. Bands 1 and 3 (Fig. 3), which were red, contained pulcherriminic acid in association with iron and protein material, as determined spectrophotometrically and by thin-layer chromatography of acid hydrolysates.

The leucine auxotroph *B. subtilis* AM-L11, when grown in medium CH containing L-leucine-*U*-^{14}C or L-leucine-*1*-^{14}C, yielded pulcherriminic acid with a molar specific activity close to twice that of the amino acid (Table 2, expt 1, 2, and 3). Similar results were obtained with suspensions of starved cells (Table 2, expt 5). The data indicated that the carbon skeletons of two L-leucine molecules were used to synthesize one molecule of pulcherriminic acid. When ^{14}C-labeled DL-leucine was used, the specific activity of pulcherriminic acid was essentially identical with the total specific activity of the D and L isomers of leucine (Table 2, expt 4). This suggested that the D isomer was not incorporated into pulcherriminic acid.

Essentially no label was found in the pulcherriminic acid produced by *B. subtilis* AM-L11 growing in medium CH to which other ^{14}C uniformly labeled amino acids were added singly (L-tyrosine, L-alanine, L-aspartic, and L-glutamic acids). These results reflected the inability of *B. subtilis* AM-L11 to synthesize L-leucine from the amino acids added and implied that, in the wild type, L-leucine is an intermediate in the synthesis of pulcherriminic acid from other amino acids.

Cycloleucyl-leucyl as an intermediate in pulcherriminic acid synthesis. Suspensions of washed cells (harvested at the beginning of pigment production) were incubated in the presence of cycloleucyl-leucyl to investigate the role of this compound in pulcherriminic acid synthesis by *B. subtilis*. Significant stimulation of pulcherriminic acid synthesis was observed when cyclo-L-leucyl-L-leucyl was the substrate (Table 3). Essentially no pulcherriminic acid was formed in the presence of cyclo-D-leucyl-L-leucyl or cyclo-D-leucyl-D-leucyl (Table 3).

Apparently the L isomer of cycloleucyl-leucyl was not degraded to L-leucine by an extracellular enzyme before entry into the cell, since leucine was not detected in the supernatant liquid of reaction mixtures incubated with cyclo-L-leucyl-L-leucyl. The results are in agreement with the hypothesis that cyclo-L-leucyl-L-leucyl is an intermediate in pulcherriminic acid biosynthesis (Fig. 1).

Bacterial cell suspensions metabolized L-leucine to pulcherriminic acid at a greater rate than cycloleucyl-leucyl (Table 3). This difference may be a reflection of the low solubility of cyclo-L-leucyl-L-leycyl or of the rate of entry of this compound into the cell, or both.

The conversion of L-leucine, or of cyclo-L-leucyl-L-leucyl, to pulcherriminic acid proceeded only in the presence of molecular oxygen (Table 3). Previous studies with growing cells had shown that O_2 was required both for the synthesis of enzymes associated with pulcherriminic acid production and for the functioning of the pathway in the presence of the required enzymes (10). The data in Table 3 indicate that the latter type of requirement is due, in part or totally, to the occurrence of reaction II (Fig. 1). It appears unlikely that O_2 is needed for the conversion of leucine to cycloleucyl-leucyl, and reaction III (Fig. 1) occurs both aerobically and anaerobically (10).

Cells incubated with L-leucine evolved CO_2 and consumed O_2, an indication that the amino acid was used catabolically as well as for the synthesis of pulcherriminic acid. The amounts of O_2 utilized for pulcherriminic acid formation from L-leucine or cycloleucyl-leucyl were not estimated.

Role of carbohydrate in pulcherriminic acid synthesis. Experiments in which *B. sub-*

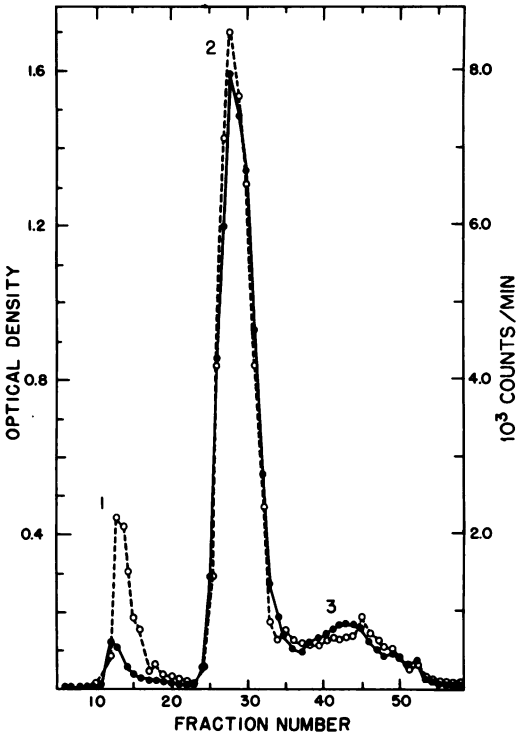

FIG. 3. *Isolation of ^{14}C-labeled pulcherriminic acid by gel filtration on a Sephadex LH-20 column. The optical density (●——●) of fractions 5 to 23 (pigmented band 1) was determined at 383 nm; of fractions 24 to 36 (band 2, consisting of pulcherriminic acid) at 410 nm; of fractions 37 to 58 (pigmented band 3) at 396 nm. The counts per minute per fraction (0.72 ml) are reported. The optical density (OD) values indicate 1/10 the OD of the eluted fractions. Radioactivity (O----O) was determined with a Packard model 3003 Tri-Carb liquid scintillation spectrometer.*

tilis AM-L11 was grown in the presence of uniformly labeled ^{14}C-starch (25 μCi in 200 ml of medium CH) indicated little or no incorporation of label in the pulcherriminic acid produced by this leucine auxotroph. These data agreed with the conclusion that leucine molecules are utilized by the organisms for pigment synthesis and indicated that the carbohydrate does not contribute to the carbon skeleton of pulcherriminic acid produced by strain AM-L11.

Since pigment synthesis began only after most of the starch disappeared from the growth medium, it was thought possible that the extracellular accumulation of a product of carbohydrate metabolism triggered or induced pulcherriminic acid production. To investigate this possibility, cells were grown in medium P until starch was no longer detected in the medium. The cells were removed by centrifugation, and the supernatant liquid was extracted with chloroform, diethyl ether, or petroleum ether. The extracts were dried in a N_2 atmosphere and dissolved in distilled water. Addition of these extracts to cells growing in

TABLE 2. *Incorporation of ^{14}C-labeled leucine into pulcherriminic acid produced by cells of Bacillus subtilis AM-L11*

Expt[a]	Labeled compound added[b]	Specific activity (counts per min per μmole)	
		L-Leucine	Pulcherriminic acid[c]
1	L-Leucine-*U*-^{14}C	147	272
2	L-Leucine-*U*-^{14}C	298	512
3	L-Leucine-*1*-^{14}C	3,330	5,900
4	DL-Leucine-*2*-^{14}C	517[d]	1,025
5	L-Leucine-*U*-^{14}C	1,000	1,740

[a] Growing cells were used in experiments 1 to 4, a starved cell suspension in experiment 5. In experiment 5, radioactivity was determined by liquid scintillation techniques. Counts were corrected for self-absorption or quenching.

[b] To medium CH in experiments 1 to 4, to the cell suspension in experiment 5 (see Materials and Methods).

[c] Purified by gel filtration (see Materials and Methods).

[d] Calculated from one-half the counts of DL-leucine-2-^{14}C.

TABLE 3. *Pulcherriminic acid synthesis by cell suspensions of Bacillus subtilis AM-L11*

Substrate	Conditions[a]	Pulcherriminic acid formed[b]
Cyclo-D-leucyl-L-leucyl	Aerobic	0.03
Cyclo-D-leucyl-D-leucyl	Aerobic	0.03
Cyclo-L-leucyl-L-leucyl	Aerobic	0.42
Cyclo-L-leucyl-L-leucyl	Anaerobic	0.00
L-Leucine	Aerobic	0.69
L-Leucine	Aerobic, then anaerobic	0.24 No increase
L-Leucine	Anaerobic	0.00

[a] Aerobic: 4 hr in air. Aerobic, then anaerobic: 1.5 hr in air, then 2.5 hr in N_2.

[b] Micromoles per reaction mixture. The values represent the average of several experiments and are corrected for endogenous metabolism. Cells incubated for 4 hr in air, without addition of substrate, produced 0.13 μmole of pulcherriminic acid per reaction mixture (3.2 ml). No pulcherriminic acid was produced anaerobically in the absence of substrate.

medium P, from which starch had been omitted, failed to stimulate pigment synthesis. However, when cells grown in a carbohydrate-free medium were suspended in the unextracted supernatant liquid mentioned above (10^9 cells/ml), pigment was produced upon incubation. Thus, it cannot be excluded that an extracellular product of carbohydrate metabolism is involved in initiating pigment production by growing cells.

Pulcherrimin-forming cultures of *Bacillus* generally contain a number of cells which do not produce pigment (5). These pigmentless mutants are readily detected since they form colorless colonies on iron-containing media (e.g., medium P including 2 g of agar/100 ml). Viable counts showed that 2×10^{-4}% of the cells in a pigmented colony of *B. subtilis* AM-L11 did not possess the ability to form pigment. Similar or higher percentages (e.g., 0.2%) of pigmentless mutants were found in pigmented broth cultures (medium PB) of strain AM-L11 at the end of the exponential phase. It was noted that pigmented colonies of strain AM-L11, growing on agar plates, hydrolyzed the starch in the medium rapidly and extensively (as shown by large clear areas around the colonies when the plates were flooded with iodine-potassium iodide solution). On the other hand, colonies of AM-L11 pigmentless mutants exhibited only limited starch-hydrolyzing ability under the same conditions. This observation suggested that the pigment-forming cells were more dependent on starch for their growth than the pigmentless mutants and that nutritional selection favoring the pigmentless population might occur during growth on media not containing starch. This possibility was tested by growing *B. subtilis* AM-L11 in medium PB from which starch was omitted and by determining the number of pigmentless and pigment-forming cells. Viable counts on P agar plates indicated that only 8.3% of the cell population consisted of pigment-producing cells at the end of growth, whereas 99.9% of the cells in the culture were of the pigment-forming type immediately after inoculation. Apparently, during growth in the starch-free medium, a drastic shift occurred in the ratio of pigment-forming to pigmentless cells; this shift resulted in a preponderance of pigmentless mutants and in the formation of small amounts of pigment.

DISCUSSION

The evidence presented in this paper indicates that *B. subtilis* produces pulcherriminic acid from L-leucine and that the organism utilizes the carbon skeletons of two leucine molecules to synthesize one molecule of the cyclic acid. Apparently cyclo-L-leucyl-L-leucyl is an intermediate in pulcherriminic acid biosynthesis. Cells growing in media containing amino acids other than leucine also produce pulcherriminic acid. However, it may be inferred from the available data that these amino acids are utilized for the synthesis of L-leucine from which pulcherriminic acid is formed.

Although growing cells produce significant amounts of pulcherriminic acid only in media including a carbohydrate (e.g., starch), carbohydrate carbon is not required for the synthesis of pulcherriminic acid molecules. It may be speculated that the accumulation of a product of carbohydrate metabolism is involved in initiating pulcherriminic acid synthesis, or that an enzyme (or enzymes) associated with carbohydrate metabolism plays a role in the formation of the cyclic acid. It is also possible that when the pigment-forming cells utilize starch for growth they are able to compete successfully with pigmentless mutants generally present in the population, whereas during growth in the absence of starch the pigmentless mutants may become numerically predominant and only small amounts of pigment are formed.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI 08248 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. Anderson, R. L., and W. A. Wood. 1962. Pathway of L-xylose and L-lyxose degradation in *Aerobacter aerogenes*. J. Biol. Chem. **237**:296–303.
3. Beijerinck, M. W. 1918. Levures chromogènes. Nouvelle réaction biologique du fer. Arch Néerl. Physiol. **2**:609–615.
4. Campbell, J. N., J. L. Nichols, and S. A. Berry. 1964. Characterization of the pigment of *Micrococcus violagabriellae*. Can. J. Microbiol. **10**:659–675.
5. Canale-Parola, E. 1963. A red pigment produced by aerobic sporeforming bacteria. Arch. Mikrobiol. **46**: 414–427.
6. Cook, A. H., and C. A. Slater. 1954. Metabolism of "wild" yeasts, I. The chemical nature of pulcherrimin. J. Inst. Brew. (London). **60**:213–217.
7. Cook, A. H., and C. A. Slater. 1956. The structure of pulcherrimin. J. Chem. Soc. 4133–4135.
8. Goto, S. 1962. Study of the mechanism of synthesis of protein molecules. J. Polym. Sci. **57**:965–974.
9. Kluyver, A. J., J. P. van der Walt, and A. J. van Triet. 1953. Pulcherrimin, the pigment of *Candida pulcher-*

rima. Proc. Nat. Acad. Sci. U.S.A. **39**:583–593.
10. Kupfer, D. G., R. L. Uffen, and E. Canale-Parola. 1967. The role of iron and molecular oxygen in pulcherrimin synthesis by bacteria. Arch. Mikrobiol. **56**:9–21.
11. MacDonald, J. C. 1963. The structure of pulcherriminic acid. Can. J. Chem. **41**:165–172.
12. MacDonald, J. C. 1965. Biosynthesis of pulcherriminic acid. Biochem. J. **96**:533–538.
13. Snell, E. E. 1957. Microbiological determination of amino acids, p. 477–492. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
14. Steele, Betty F., H. E. Sauberlich, M. S. Reynolds, and C. A. Baumann. 1949. Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081. J. Biol. Chem. **177**:533–544.
15. Uffen, R. L., and E. Canale-Parola. 1966. Temperature-dependent pigment production by *Bacillus cereus* var. *alesti*. Can. J. Microbiol. **12**:590–593.
16. Uffen, R. L., and E. Canale-Parola. 1969. Isolation of pulcherriminic acid from cultures of *Bacillus cereus* var. *alesti*. Zeitsch. Allg. Mikrobiol. **9**:231–233.
17. van der Walt, J. P. 1952. On the yeast *Candida pulcherrima* and its pigment. 's-Gravenhage, Delft.