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Production of a Molybdenum-Coordinating Compound by *Bacillus thuringiensis*

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Bacillus thuringiensis (ATCC 10792) produces a molybdenum reactive compound (given the trivial name chelin) during growth on iron-deficient medium. This compound accumulates in the culture medium in direct relation to the amount of L-arginine added and reaches a maximum concentration 24 to 48 h after the stationary phase of growth. Chelin absorbs light in the ultraviolet region with absorption maxima at 315 and 248 nm and minima at 284 and 240 nm. Chelin reacts with Na_2MoO_4, but not with $Mo_2O_4(H_2O)_6^{2+}$, to form a bright yellow molybdo-chelin complex which absorbs light with an absorption maximum at 330 nm, a minimum at 288 nm, and shoulders at 255 and 400 nm. The differential absorption of molybdo-chelin versus chelin at 425 nm can be used to quantify chelin. This differential absorbance is linear with increasing concentrations of Na_2MoO_4 and was used to calculate the molar extinction coefficient of molybdo-chelin at 425 nm ($\epsilon \sim 6{,}200$). Chelin binds MoO_4^{2-} to form a complex (molybdo-chelin) which migrates as a single band and elutes as a single peak, during acrylamide gel electrophoresis and Sephadex G-15 gel filtration. Molecular weight determinations using Sephadex G-15 gel filtration resulted in an estimated molecular weight of 550 for chelin and an estimated molecular weight of 760 for molybdo-chelin. The peptide nature of chelin is indicated by its positive ninhydrin reaction on thin-layer chromatography plates and by the presence of amino acids in acid-hydrolyzed samples. The major amino acid residues detected were threonine, glycine, and alanine.

Molybdenum serves a catalytic function in both nitrogenase and nitrate reductase and is a required trace element for organisms growing on either dinitrogen or nitrate as a sole nitrogen source. The coordination chemistry of molybdenum in these enzymes is at present uncertain; however, model compounds of molybdenum organic complexes have been synthesized in attempts to mimic the catalytic properties of molybdenum in nitrogenase (5, 15, 16). One of these complexes is formed by the coordination of molybdenum to glutathione (16). Glutathione and the molybdenum-containing peptide isolated from nitrogenase (4) are the only peptides known to coordinate molybdenum.

The preliminary characterization (7, 8) of a low-molecular-weight molybdenum factor involved in the in vitro restoration of nitrate reductase in *Neurospora crassa nit-1* (6, 10) stimulated us to study the hypothesis that microorganisms produce low-molecular-weight molybdenum-coordinating compounds. An analogous phenomenon is the microbial production of small molecules called siderochromes, which coordinate and transport iron (12, 13). This paper reports the discovery of a compound produced by *Bacillus thuringiensis* which coordinates molybdenum.

MATERIALS AND METHODS

Organism and growth. Nutrient broth yeast extract medium was inoculated with a loop of a spore suspension of *B. thuringiensis* (ATCC 10792) and grown at 23 C for 18 h (8). Five milliliters of the nutrient broth yeast extract culture were transferred to 1 liter of the iron-deficient medium described previously (9) which routinely contained 3 g of L-arginine per liter. Cells were removed from the culture by centrifugation at 10,000 × *g* for 20 min. The resulting culture supernatant was used as a source of molybdenum-coordinating compound. Cell growth was determined by measuring the absorbance at 600 nm.

Chelin assay. The increase in absorbance at 425 nm after the addition of 0.05 ml of a 10^{-2} M Na_2MoO_4 solution to 1 ml of culture supernatant was taken as a measure of chelin. Samples having an optical density (OD) at 425 nm greater than 0.600 were diluted and reassayed. No increase in absorbance at 425 nm was observed when the same amount of Na_2MoO_4 was added to 1 ml of the sterile culture medium or when the same amount of NaCl was added to culture supernatant.

Isolation of chelin. The procedure of Neilands (11) for the isolation of ferrichrome was used as modified below. Samples of culture supernatant were adjusted to pH 9.0 with 1 N NaOH before the addition of $(NH_4)_2SO_4$ to make an 80% saturated solution (56.1 g/100 ml). The solution was adjusted to pH 6.5 and the precipitate was removed by centrifugation before extracting the supernatant with 0.1 volume of chloroform-phenol (1:1, vol/vol) for 2 min. The organic phase was removed after separation by centrifugation. At this stage, the molybdenum-coordinating compound could be titrated out of the organic phase by adding excess Na_2MoO_4 in aqueous solution and 10 volumes of ethyl ether. However, this procedure resulted in a product which contained phenol. The preferred approach is to precipitate the compound out of the organic phase by adding 2.5 volumes of ethyl ether followed by washing the precipitate twice with chloroform. The washed precepitate is soluble in methanol and water and insoluble in acetone, chloroform, and ethyl ether. The molybdenum-coordinating compound was further purified by Sephadex G-15 gel filtration on a column (0.9 by 25 cm) using distilled water as the developing solvent. The molybdenum-reactive material eluted after the void volume as a single peak. Purified material was used directly or stored at −20 C to prevent microbial decomposition.

Acrylamide gel electrophoresis. Samples (100 μl) were electrophoresed on 15% acrylamide gels (3) at 1.5 mA/tube for 1.5 h in 0.005 M tris(hydroxymethyl)-aminomethane-glycine buffer, pH 8.3. Gels were stained for molybdenum by incubating the gel for 20 min in 7 ml of 3.8 N HCl followed by the addition of 3.0 ml of dithiol reagent (1) and continued incubation at 35 C for 40 min. The resulting green-staining molybdenum band was stable for 1 month when stored in 7% (vol/vol) acetic acid.

Thin-layer chromatography. Samples of the Sephadex peak with and without molybdenum were spotted on Eastman Chromagram standard silica gel sheets and developed with isopropyl alcohol-water (70:30) in the Eastman chamber plate apparatus. The material was visulized by spraying with a solution of either Na_2MoO_4 (10^{-2} M), ninhydrin, or the dithiol reagent used for the detection of molybdenum (1).

Hydroxylamine and amino acid analysis. The molybdenum-reactive fractions from the Sephadex G-15 column were concentrated and then hydrolyzed under a nitrogen atomosphere in 6 N HCl for 3 h at 140 C. The resultant hydrolysate was analyzed for amino acids using a Beckman amino acid analyzer. Hydroxylamine was estimated by the procedure of Yashphe et al. (17).

RESULTS

Production and isolation of chelin. The presence of molybdenum-reactive compounds in culture filtrates of *B. thuringiensis* was dependent on the phase of growth and the concentration of L-arginine added to the medium. The reaction between Na_2MoO_4 and components of the culture filtrate was first detected in the late exponential phase and was maximal 24 to 48 h after the onset of the stationary phase of growth. No attempts were made to alter the basic medium except an investigation of the relationship between L-arginine concentration and the yield of molybdenum-reactive component(s). Increasing concentration of L-arginine added to the iron-deficient medium (9) increased the yield of molybdenum-reactive component(s) (Table 1), as is indicated by the increasing amount of OD_{425}-absorbing material. This observation led to the routine growth of *B. thuringiensis* in media containing 3.0 g of L-arginine per liter. Culture filtrates from 92-h cultures (ΔOD_{425} from 0.169 to 0.548) were used as the source of chelin for the isolation and characterization experiments described below.

Isolation of the compound from culture filtrates was attained by extraction into chloroform-phenol (1:1), precipitation by ether, and Sephadex G-15 gel filtration. Approximately 25% of the chelin was recovered in the ether precipitate. Chelin eluted from the Sephadex column as a single peak and was used in the chemical characterization studies. Thin-layer chromatograms of this fraction developed in isopropyl alcohol-water (70:30) revealed a major ninhydrin-positive spot, R_f = 0.7, which also turned yellow if sprayed with a dilute solution of Na_2MoO_4.

Molecular weight determination. An estimation of the molecular weight of the chelin was obtained by Sephadex gel filtration on G-15 (Fig. 1). The chelin eluted as a major peak with a calculated molecular weight of 550. Molybdo-chelin, prepared by adding excess Na_2MoO_4 to chelin preparations, had a calculated molecular weight of 760. The fractions which contained molybdo-chelin were readily identified by

TABLE 1. *Effect of arginine concentration on the production of molybdenum-coordinating compound(s) by Bacillus thuringiensis*[a]

Time of growth (h)	L-Arginine (g/liter)	Cell density of culture (OD_{600}/ml)	Culture filtrate plus Na_2MoO_4 (ΔOD_{425}/ml)
72	0.5	6.99	0.065
	3.0	11.23	0.151
	6.0	7.13	0.234
92	0.5	6.61	0.074
	3.0	12.37	0.169
	6.0	9.00	0.303

[a] The amount of Na_2MoO_4-reactive compound(s) in the culture filtrate was measured as the difference between the absorbance at 425 nm in the presence and absence of Na_2MoO_4.

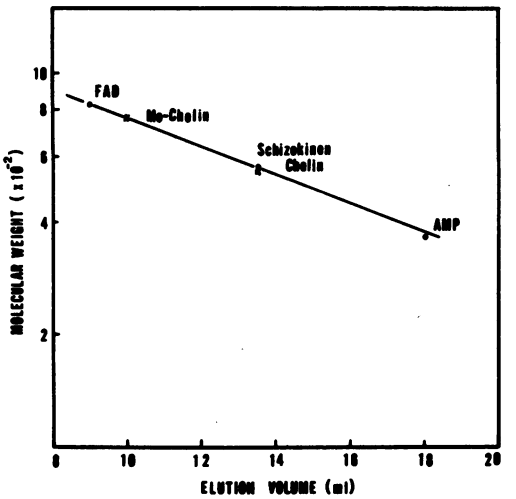

FIG. 1. *Molecular weight estimation of chelin and molybdo-chelin on Sephadex G-15. The Sephadex G-15 column was standardized using blue dextran, flavin adenine dinucleotide (FAD), schizokinen purified from B. megaterium filtrates (9), and adenosine monophosphate (AMP). Separate runs were made using 413 nmol of chelin/0.1 ml in the presence and absence of Na_2MoO_4. The elution profiles of chelin and molybdo-chelin were determined spectrophotometrically at 425 nm (with and without added Na_2MoO_4) and shown to be symmetrical.*

their absorption of 425 nm and by the absence of a significant absorption increase at 425 nm after the addition of Na_2MoO_4. When a limiting amount of Na_2MoO_4 was added to the chelin sample before sephadex gel filtration, chelin and molybdo-chelin eluted in two distinct peaks. Molybdenum eluted with the larger complex which absorbed at 425 nm and which showed no significant increase in absorbance at 425 nm upon the addition of Na_2MoO_4. The increase in the molecular weight of molybdo-chelin over chelin is indicative of complex formation and suggests that 1 mol of MoO_4^{-2} (163 daltons) reacts with 1 mol of chelin.

Special analysis of chelin. Aqueous solutions of chelin are pale yellow and have absorption maxima at 315 and 248 nm and minima at 240 and 284 nm. Aqueous solutions of molybdo-chelin are bright yellow and have an absorption maximum at 330 nm, a minimum at 288 nm, and shoulders at 255 and 400 nm (Fig. 2). Therefore, reaction of chelin with Na_2MoO_4 shifts the adsorption maxima and minima to longer wavelengths and increases the absorption between 310 and 525 nm. The increase in absorption above 330 nm is greatly diminished upon acid treatment (to pH 1.5). Titration of chelin with Na_2MoO_4 demonstrated a linear relationship between the increase in absorbance at 425 nm and the concentration of Na_2MoO_4 (Fig. 3). At 425 nm the extinction coefficient of molybdo-chelin was calculated as 6,200. A preliminary infrared spectrum of chelin showed a

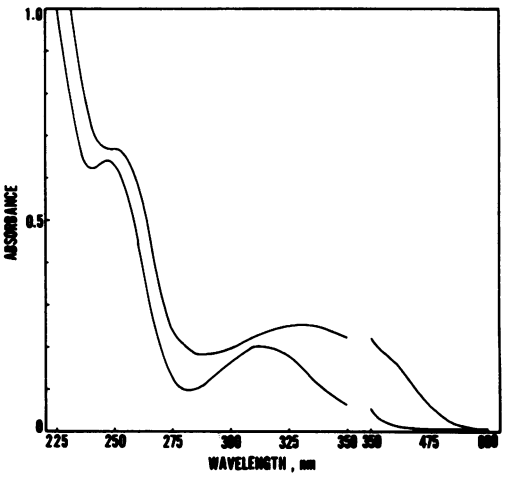

FIG. 2. *Absorption spectra of chelin in the presence and absence of sodium molybdate. The peak tubes containing molybdenum-reactive material from the Sephadex G-15 column were pooled. An aliquot was diluted in distilled water and scanned in a Zeiss DMR 21 spectrophotometer with water in the reference cuvette (bottom curve). The sample, containing 20 nmol of chelin (pH 6.5), was rescanned after the addition of Na_2MoO_4 (3.3×10^{-4} M final concentration) to both the sample and reference cuvettes (top curve).*

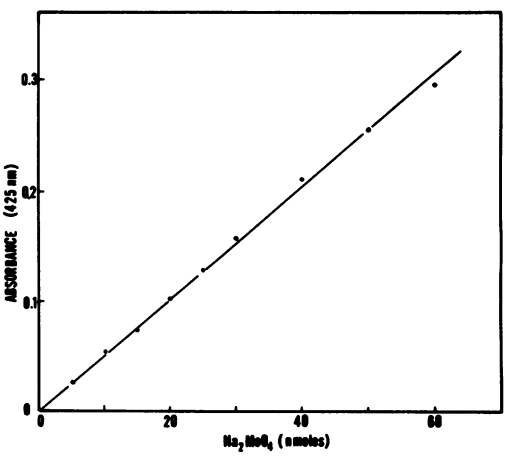

FIG. 3. *Titration of chelin with sodium molybdate. Purified chelin was diluted in distilled water and the absorbance was measured as a function of the amount of Na_2MoO_4 added. The values reported are corrected for dilution and the initial absorption of chelin at 425 nm (0.037).*

spectrum similar to the one reported for the molybdenum peptide isolated from nitrogenase (4). Addition of Na_2MoO_4 to the chelin sample did not change significantly the infrared spectrum. Electron paramagnetic resonance spectra of molybdo-chelin (2.2 μmol/ml) showed no significant signal in the g = 2.0 region when the sample was maintained either at neutral or acid pH indicating the absence of a MoV species. The absence of MoV in molybdo-chelin is further suggested by the observation that chelin does not react with the MoV species, $Mo_2O_4(H_2O)_6^{+2}$.

Acrylamide gel electrophoresis. To detect molybdenum-reactive compounds in extracted culture filtrates, a rapid technique using acrylamide gel electrophoresis was developed. Chelin was extracted from the chloroform-phenol extract either by adding 10 volumes of ether and 1 volume of water, or molybdo-chelin was extracted by titrating the chelin out of the chloroform-phenol-ether phase with small volumes of an aqueous solution of Na_2MoO_4 (10^{-2} M). Disc gel electrophoresis (Fig. 4) of chelin revealed a pale yellow band which when treated with Na_2MoO_4 produced an intense yellow color. Electrophoresis of molybdo-chelin and subsequent staining with the molybdenum-specific dithiol reagent revealed two bands; the slower band corresponded to the intense yellow band observed before staining and the faster moving band corresponded to the Na_2MoO_4 standard (treated in an identical manner to the chelin). Both chelin and molybdo-chelin migrated to the anode during these electrophoresis experiments (pH 8.3) and thus are negatively charged. This disc gel electrophoresis method is a rapid technique for detecting molybdenum-reactive compounds and further demonstrates that chelin forms a complex with molybdenum.

Chemical analysis of chelin. Chemical analysis of purified chelin (Sephadex G-15 fraction) indicates that this compound contains three major amino acids residues (Table 2). Amino acid analysis revealed the presence of threonine, glycine, and alanine together with trace amounts of other amino acids. We were unable to detect basic amino acids in the sample. Among the amino acids present in trace amounts, phenylalanine was the only aromatic amino acid. Hydroxylamine was not detectable in the acid hydrolysate.

Biological activity of chelin. A possible role for chelin in biological systems is the transport of molybdenum into cells and/or the direct involvement of molybdo-chelin as a component of molybdenum-containing enzymes. The latter possibility is easily tested by the in vitro restoration of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-nitrate reductase assay (6–8, 10). Attempts were made to substitute molybdo-chelin for the dialyzable factor donated by cell-free extracts of *B. thuringiensis* to the in vitro restoration of NADPH-nitrate reductase (8) in extracts of *N. crassa nit-1*. Molybdo-chelin did not restore NADPH-nitrate reductase activity when added to preparations of *N. crassa nit-1* which were active in the restoration of NADPH-nitrate reductase when mixed with either acid treated xanthine oxidase or extracts of *B. thuringiensis*.

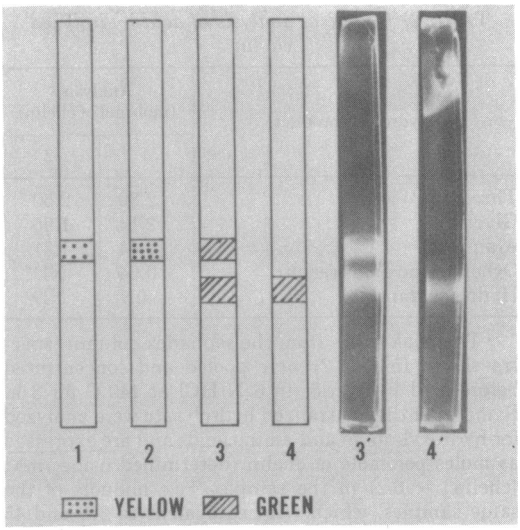

FIG. 4. *Acrylamide gels of chelin and molybdo-chelin. Samples (100 μliters) were electrophoresed as described in Materials and Methods. The gels diagramed and designated by numbers 1 to 4 are as follows: (1) chelin extracted from the chloroform-phenol-ether phase by water, unstained; (2) chelin extracted from the chloroform-phenol-ether phase with 10^{-3} M Na_2MoO_4, unstained; (3) same as sample 2 except stained for molybdenum; and (4) electrophoresis of 10^{-3} M Na_2MoO_4 (treated in an identical manner to the Na_2MoO_4 used in the extraction of sample 2 minus the culture filtrate) stained for molybdenum. Permanent records of the gels were made on Ektachrome 35-mm film. Black and white positives of gels 3 and 4 are labeled as 3' and 4', respectively. The yellow bands in gels 1 and 2 were not reproducible by this photographic method and therefore are presented as diagrams.*

DISCUSSION

The evidence presented demonstrates that *B. thuringiensis* produces a low-molecular-weight peptide-containing compound which reacts

TABLE 2. *Chemical analysis of acid-hydrolyzed chelin*[a]

Hydrolysis product	Analysis (mol/mol of chelin)	
	1	2
Threonine	2.80	2.50
Glycine	2.14	1.95
Alanine	1.44	1.31
Other amino acids (each)	<0.04	ND[b]
Hydroxylamine	0	ND[b]

[a] The peak tubes from the sephadex column (spectra shown in Fig. 2) were pooled and concentrated before acid hydrolysis in 6 N HCl at 140 C for 3 h. Samples of the neutralized hydrolysate were analyzed for hydroxylamine and amino acids and are expressed as moles per mole of chelin (determined using ϵmM [chelin] = 6.2) in the sample. Two aliquots of the same samples, which were equivalent to 270 and 45 nmol of chelin, respectively, were analyzed on a Beckman amino acid analyzer.

[b] ND, Not determined.

with Na_2MoO_4 to produce a molybdenum-peptide complex. Chelin is produced by *B. thuringiensis* when it is grown on the iron-deficient medium used for the production of schizokinen by *B. megaterium* (9). Although the mechanism is not understood, these organisms produce more chelin or schizokinen, respectively, when L-arginine is added to the growth medium. Complex formation between chelin and Na_2MoO_4 is demonstrated by the decreased mobility of molybdenum and its comigration with chelin during electrophoresis, the elution of molybdenum with the 760-dalton form of chelin during Sephadex gel filtration, and by the increase in the molecular weight of chelin after reaction with Na_2MoO_4. The molecular weight difference between molybdo-chelin and chelin, and the titration curve for the reaction of chelin with Na_2MoO_4, both indicate that 1 mol of chelin reacts with 1 mol of Na_2MoO_4. Assuming that chelin reacts on a one-to-one basis with Na_2MoO4, the estimated molar extinction coefficient for molybdo-chelin, based on moles of Na_2MoO4, was calculated to be 6,200 at 425 nm. The peptide nature of chelin is demonstrated by its reaction with ninhydrin on thin-layer chromatography plates and by the amino acid analysis which demonstrates the presence of threonine, glycine, and alanine as the major amino acid residues of chelin. The other amino acid residues, detected in trace amounts, are presumably due to sample contamination. The strong absorption band at 248 nm observed in aqueous solutions of chelin is indicative of the presence of an aromatic moiety. Further investigations are in progress to determine the nature of the aromatic material and to determine if this absorption is due to an inadequacy in the purification procedure or if it is due to the presence of an aromatic moiety bound to the chelin peptide.

The isolation of chelin is the first demonstration of the production and excretion of a molybdenum-coordinating peptide by a microorganism. Interest in the existence of such compounds has been stimulated by the genetic experiments (14) on the *Aspergillus nidulans* NADPH-nitrate reductase (EC 1.6.6.2) and the in vitro restoration of NADPH-nitrate reductase in *N. crassa nit-1* (6–8, 10). These investigations suggest that molybdenum-containing enzymes possess a "molybdenum cofactor". The chelin will not replace cell-free extracts of *B. thuringiensis* (8) in the in vitro restoration of NADPH-nitrate reductase. Chelin has properites which are also different from the molybdenum-peptide isolated from nitrogenase (4). The molybdenum-peptide is larger than chelin, contains different amino acid residues, and yields a paramagnetic Mo species (identified as MoV) upon acid treatment (4).

Although the chemistry of the coordination of molybdenum to chelin has not been determined, it is interesting to note that chelin coordinates the highest oxidation state of molybdenum. Molybdate is the form of molybdenum prevalent in aqueous environments, since the lower oxidation states of molybdenum (III, IV, V) are air sensitive (2). Therefore, one would expect organisms to produce compounds which coordinate molybdate and not lower oxidation states of molybdenum if such coordination complexes were necessary for growth. Chelin meets this expectation since it complexes with the anion molybdate and not the cation, $Mo_2O_4(H_2O)_6^{+2}$. The oxidation state of molybdenum does not appear to change during complex formation since no paramagnetic molybdenum electron paramagnetic resonance signal was observed indicating the absence of a single MoV atom in the complex. The presence of a nonparamagnetic di-molybdenum species in molybdenum-chelin is tentatively ruled out because of the one-to-one stoichiometry of the reaction.

The characteristics of chelin make this compound more analogous to the siderochromes than to a component of molybdo-enzymes. Like siderochromes, chelin is excreted into the medium and coordinates a trace metal necessary for growth under specific conditions. Since siderochromes are involved in the transport of iron (13), it is possible that chelin may serve a

transport function for molybdenum in *B. thuringiensis*. Even though the physiological role of chelin is as yet undetermined, chelin is of significant interest because it can serve as a model for studying the coordination chemistry of molybdenum in a naturally occurring peptide which lacks sulfur-containing amino acids.

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