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Stimulation of Leucomycin Production by Magnesium Phosphate and Its Relevance to Nitrogen Catabolite Regulation

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Addition of magnesium phosphate [$Mg_3(PO_4)_2 \cdot 8H_2O$] to a complex medium or to an ammonium ion-containing, chemically defined medium stimulated leucomycin production by *Streptomyces kitasatoensis*. Ammonium ions in high concentrations inhibited leucomycin production, but their limitation by magnesium phosphate led to the high production of the antibiotic.

Biosynthesis of antibiotics is often regulated by carbon catabolites, nitrogen catabolites, phosphates, and other metabolites (2). High production of these products may be achieved when the producing organisms are cultivated under the conditions which favor their escape from one or more of such regulators. The general techniques which have been employed for this purpose are the use of media containing slowly utilized carbon or nitrogen sources or both and a continuous feeding of suitable carbon sources at low concentrations (2). In addition, the optimalization of cultural conditions (pH, temperature, oxygen supply, etc.) and strain improvement may also lead to such desirable results.

Several papers have described the inhibition of the formation of antibiotics by ammonium ions and by rapidly utilized nitrogen sources (1, 3, 4, 9, 11, 12, 14). Although the exact biochemical basis of the inhibition is not available, the phenomenon is called nitrogen catabolite regulation by analogy with carbon catabolite regulation, in which carbon catabolites are known to inhibit antibiotic formation (2). To date, the importance of nitrogen catabolite regulation and the methods to reduce its effect have not been assessed adequately.

Recently, it was shown that microbial conversion of glycine to L-serine by *Nocardia butanica* was stimulated by magnesium phosphate (13). The stimulation seemed to be related to the reduction of inhibition by ammonia in the L-serine formation system. In view of this observation, it was of interest to determine whether magnesium phosphate would have a similar effect on the synthesis of antibiotics.

A novel and simple cultivation method was subsequently found by which magnesium phosphate reduced ammonium ion concentration in the leucomycin (7) (Fig. 1) fermentation medium and resulted in a severalfold increase of the antibiotic. The stimulation seems to be due to relaxation of the nitrogen catabolite regulation of the leucomycin biosynthesis.

MATERIALS AND METHODS

Microorganism. *S. kitasatoensis* KA-429, a mutant strain which produces leucomycins A_1 and A_3 as major components in a complex medium, was used.

Method of cultivation. Mycelia and spores of the microorganism were transferred into a test tube (2 by 20 cm) containing 10 ml of a fermentation medium and incubated at 27°C for 2 to 5 days with reciprocal shaking (240 strokes/min). Two kinds of fermentation media were used. Chemically defined medium (pH 7.5) contained 3% glycerol, 0.5% glucose, 1% ammonium lactate, 0.1% $MgSO_4 \cdot 7H_2O$, 0.001% K_2HPO_4, 0.5% $CaCO_3$, and a trace metal solution (1 ml/liter) containing (each at 1 g/liter) $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 2H_2O$ and $CuSO_4 \cdot 5H_2O$, and 0 or 1% $Mg_3(PO_4)_2 \cdot 8H_2O$. Complex medium (pH 7.3) consisted of 2% glucose, 0.5% peptone, 0.3% dried yeast cells, 0.5% meat extract, 0.5% NaCl, 0.3% $CaCO_3$, and 0 or 1% $Mg_3(PO_4)_2 \cdot 8H_2O$.

Determination of leucomycin. A culture broth was adjusted to approximately pH 7.5 with solid ammonium bicarbonate and centrifuged (2,500 rpm, 10 min), and the supernatant was extracted once with an equal volume of ethyl acetate. The ethyl acetate layer was used for the assay of leucomycin by a conventional paper disk method, using leucomycin A_3 as standard and *Sarcina lutea* PCI 1001, which was seeded in nutrient agar and incubated at 37°C for 20 h, as test organism. The mean values of duplicate assays in duplicate experiments are shown.

Analysis of leucomycin components. The composition of the leucomycins was analyzed with a Shimadzu double-beam chromatogram scanner (model CS-910) at 232 nm after thin-layer chromatographic separation on silica gel (0.25 mm; no. 5723, E. Merck Darmstadt) with benzene-acetone (1:1).

Other analytical methods. Ammonium ion concentration in the supernatant of the culture broth was assayed by the indophenol method (10), and soluble inorganic phosphate was assayed by the Allen method as modified by Nakamura (6). The sediment was

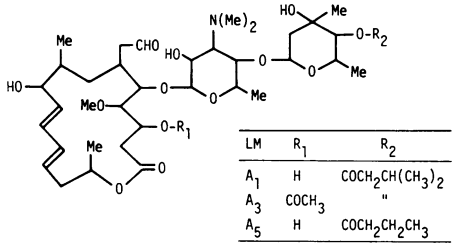

FIG. 1. *Structures of leucomycins A_1, A_3, and A_5.*

in three 10-ml portions of water and suspended in 6 ml of 1 N hydrochloric acid. After a vigorous stirring, 4 ml of 1 N sodium hydroxide was added. The solution was centrifuged (2,500 rpm, 10 min), and the packed mycelium was used to determine the amount of the mycelial growth. The amount was expressed in percentage of the packed mycelial volume against the volume of the culture broth from which the mycelium was sedimented. Dry mycelial weight was measured after washing the packed mycelium with water and drying overnight at 60°C. The supernatant of the above centrifugation containing acid-soluble materials was used for the assay of insoluble ammonia and insoluble inorganic phosphate by the methods described above.

RESULTS

Effect of addition of magnesium phosphate on leucomycin production. When *S. kitasatoensis* strain KA-429 was cultivated in a complex medium supplemented with 1% magnesium phosphate, it produced 3,800 μg of leucomycin per ml at day 2 of cultivation, which was five times more than was produced without magnesium phosphate (700 μg/ml; Fig. 2). A sevenfold increase of the leucomycin titer also occurred in a chemically defined medium (Fig. 3A). One percent of magneisum phosphate was suitable for leucomycin production, but higher concentrations were inhibitory in both media. Table 1 shows that a most significant stimulation took place when magnesium phosphate was added at an early-to-middle logarithmic phase of growth. The addition at later stages gave smaller or no effect. Mycelial growth increased notably in the chemically defined medium supplemented with magnesium phosphate; it nearly doubled as estimated by dry mycelial weight (data not shown). In both cases, the composition of the major components of the leucomycins (leucomycins A_1 and A_3 in the complex medium and leucomycins A_3 and A_5 in the chemically defined medium) was not changed significantly by the addition of magnesium phosphate.

Of other magnesium salts and inorganic phosphates tested, magnesium pyrophosphate and sodium phosphomolybdate had almost the same stimulatory effect on leucomycin production as magnesium phosphate (Table 2). Simultaneous addition of magnesium sulfate and potassium phosphate was beneficial essentially to the same extent. On the other hand, a single addition of magnesium sulfate, magnesium chloride, or sodium or potassium phosphate was rather inhibitory (data not shown).

Effect of magnesium phosphate on ammonium ion concentration. The stimulatory effect of magnesium phosphate in the chemically defined medium was studied further to determine whether this compound is involved in nitrogen and phosphate regulation by comparing the ammonia and inorganic phosphate levels in the presence and absence of magnesium phosphate (Fig. 3B). When magnesium phosphate was added, soluble inorganic phosphate increased with a corresponding decrease of insoluble inorganic phosphate. Ammonium ion concentration decreased rapidly as the pH value was lowered, and mycelial growth increased. On the other hand, insoluble ammonia at zero time was fairly high, which was consistent with the decrease of the initial soluble ammonia described above. The insoluble ammonia increased further until 30 h, when it reached a plateau. It decreased steeply in a later period without a concomitant increase of soluble ammonia, suggesting that the solubilized ammonia was immediately utilized by the mycelia. Leucomycin continued to increase during these changes, which did not occur in the absence of magnesium phosphate.

Effect of ammonium ions on leucomycin production. When ammonium sulfate was added to a chemically defined medium containing L-lysine (1% as hydrochloride) as the sole nitrogen source with no magnesium phosphate,

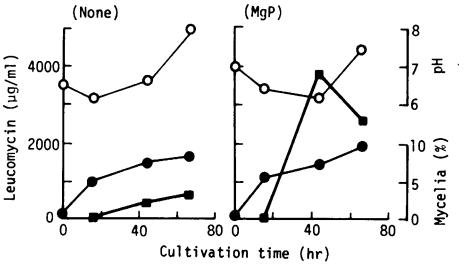

FIG. 2. *Time courses of leucomycin production in a complex medium in the presence and absence of magnesium phosphate (MgP). S. kitasatoensis strain KA-429 was cultivated at 27°C in a series of test tubes, each containing a complex medium with or without magnesium phosphate. At various times the tubes were withdrawn, and the culture broths were treated as described in the text and assayed for mycelial growth (●), pH (○), and leucomycin (■). Mean values of leucomycin titers obtained by duplicate assays with duplicate cultures are shown.*

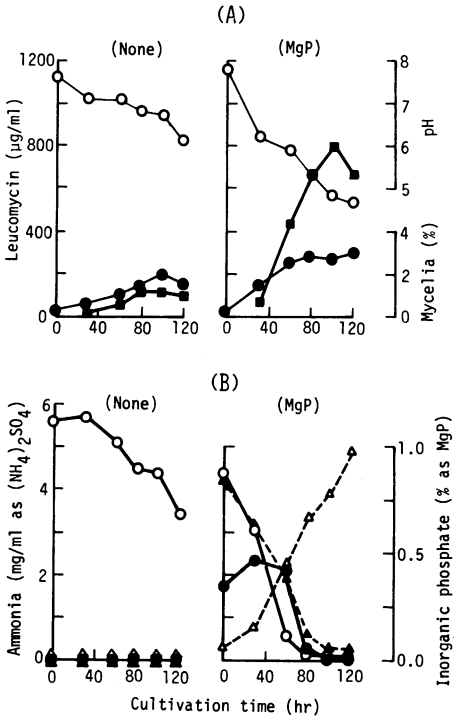

FIG. 3. *(A) Time courses of leucomycin production in a chemically defined medium with and without magnesium phosphate (MgP). (B) Time courses of concentrations of soluble and insoluble ammonia and soluble and insoluble inorganic phosphate in the presence and absence of magnesium phosphate. In (A) S. kitasatoensis strain KA-429 was cultivated in a chemically defined medium, and the culture broths of various cultivation times were assayed for leucomycin (■), mycelial growth (●), and pH (○) as described in the legend to Fig. 2. In (B), each of the same broths as used above was fractionated into supernatant and precipitated fractions as described in the text. Supernatant fractions were assayed for soluble ammonia (○) and soluble inorganic phosphate (△). Precipitated fractions were assayed for insoluble ammonia (●) and insoluble inorganic phosphate (▲) after solubilization.*

leucomycin production was severely inhibited (Table 3). The ammonium ion concentration causing 50% inhibition of the leucomycin was approximately 2 mM (0.15 mg/ml as ammonium sulfate).

To examine the effect of the time of ammonium ion addition on leucomycin production, ammonium sulfate was added to the L-lysine-containing medium at various times of cultivation. Table 4 shows that a most significant inhibition of leucomycin production was observed when the ammonium ions were added in the period of mycelial growth. Although some variations were observed in the leucomycin titers, the inhibitory effect of ammonium ions, as well as the stimulation by magnesium phosphate, was essentially identical.

An analogous feeding experiment revealed that the amount of inorganic phosphate required for 50% inhibition was ca. 90 mM, equivalent to nearly 2% magnesium phosphate, indicating a weak phosphate regulation in the leucomycin biosynthesis.

Effect of amino acids as nitrogen sources on leucomycin production. The simultaneous occurrence of the enhancement of leucomycin production and the decrease of ammonium ion concentration shown in Fig. 3 suggested that a limited supply of ammonium ions is more favorable for leucomycin production. To confirm this, various amino acids, which were expected to allow a slow supply of ammonia (1, 3), were examined as the sole nitrogen sources (Table 5). Among the amino acids tested, L-valine, L-leucine, L-lysine, L-histidine, L-asparagine, and L-proline increased the production of leucomycin, while L-alanine, L-glutamic acid, L-arginine, and L-isoleucine yielded the same amounts as ammonium ions. The enhancement of leucomycin production with L-valine and L-leucine as nitrogen sources may be related to the fact that they were also utilized as precursors of the 4″ side chain of leucomycin (5).

DISCUSSION

When the fermentation media were supplemented with magnesium phosphate, leucomycin production by *S. kitasatoensis* increased severalfold without significantly affecting the ratio of the leucomycin components. The time course study of the ammonia content in the presence of magnesium phosphate (Fig. 3B) revealed that

TABLE 1. *Effect of the time of magnesium phosphate addition on leucomycin production*[a]

Time of magnesium phosphate addition (h)	Growth (%) at the time of magnesium phosphate addition	Leucomycin produced (μg/ml)	Growth after 95 h	pH
0	—[b]	285	+++	6.0
17	2	600	+++	5.5
42	3	350	+++	5.8
64	4	280	++	6.6
72	4	295	++	6.8
89	4	275	++	7.6
No addition	—	200	+	6.8

[a] *S. kitasatoensis* strain KA-429 was cultivated in the chemically defined medium, from which magnesium phosphate was omitted. At the time indicated, 1% magnesium phosphate was added, and each culture was incubated for a total of 95 h and then assayed.

[b] —, Means not applicable.

TABLE 2. *Effect of magnesium salts, inorganic phosphates, and pyrophosphates on leucomycin production in a chemically defined medium*

Compound added[a]	Concn (%)	Leucomycin produced (μg/ml)	Growth	pH
None	—	130	+	6.9
$Mg_3(PO_4)_2 \cdot 8H_2O$[b]	1.0	240	++	4.9
$Mg_2P_2O_7$	1.0	220	++	5.0
$MnHPO_4 \cdot H_2O$	1.0	122	+++	4.9
$Zn_3(PO_4)_2$	1.0	166	++	4.8
$Ca_3(PO_4)_2$	1.0	5	(±)	7.6
$Na_3PO_4 \cdot 12MoO_3 \cdot XH_2O$	1.0	200	++	4.7
K_2HPO_4 (0.5%) + KH_2PO_4 (0.2%) + $MgSO_4 \cdot 7H_2O$ (0.5%)		195	+++	5.2

[a] Magnesium phosphate and the other compounds indicated were added to the chemically defined medium. *S. kitasatoensis* strain KA-429 was cultivated for 4 days as described in the text.
[b] Magnesium phosphate.

TABLE 3. *Inhibition of leucomycin production by ammonium ions*[a]

Ammonium sulfate added (mg/ml)	Leucomycin produced (μg/ml)	Mycelial growth	pH
0	125	++	6.6
0.1	100	++	6.5
0.2[b]	40	++	6.7
0.5	26	++	6.8
1.0	28	++	7.0
2.0	1	(±)	7.4

[a] Ammonium lactate in the chemically defined medium was replaced by 1% L-lysine, and ammonium sulfate was added as indicated. *S. kitasatoensis* strain KA-429 was cultivated for 5 days.
[b] Approximately 3 mM.

TABLE 4. *Effect of the time of the ammonium ion addition on leucomycin production*[a]

Ammonium sulfate added at (h):	Growth at ammonium sulfate addition	Leucomycin produced (μg/ml)	Growth after 95 h	pH
0	−	210	+++	6.7
17	+	185	+++	6.6
42	++	190	+++	6.7
64	+++	220	+++	6.5
72	+++	235	+++	6.6
No addition		305	+++	6.6

[a] *S. kitasatoensis* strain KA-429 was cultivated in a chemically defined medium, in which ammonium lactate was replaced by 1% L-lysine. At the time indicated, ammonium sulfate (1 mg/ml) was added. Leucomycin was determined after a total of 95 h of incubation.

soluble ammonia decreased to a level of less than 1 mg/ml, whereas insoluble ammonia, which is absent in a usual condition, amounted to 30 to 40% of the total ammonia. These results show that magnesium phosphate was solubilized by autoclaving or in acidic conditions and that it then complexed the ammonium ions in the culture broth, thus causing the decrease of ammonium ions in the medium. The ammonia, once insolubilized by magnesium phosphate, was gradually released and utilized by the mycelium.

The biosynthesis of several antibiotics, such as streptomycin (3), cephamycin (1), novobiocin (11), candihexin (4), fusidic acid (9), or trihydroxytoluene (14), was suggested to be subject to nitrogen catabolite regulation, and the production of oleandomycin, a macrolide antibiotic, was shown to be inhibited by high concentrations of ammonium ions (12), but the exact mechanism of the inhibition is not known. The production of leucomycin by *S. kitasatoensis* studied here was similarly inhibited in the pres-

TABLE 5. *Effect of amino acids as nitrogen sources on leucomycin production*[a]

Nitrogen source (each at 1%)	Leucomycin produced (μg/ml)	Mycelial growth	pH
L-Valine	560	++	6.8
L-Leucine	300	++	6.3
L-Lysine[b]	340	++	6.4
L-Histidine	350	++	6.2
L-Asparagine	280	++	6.6
L-Proline	190	++	6.4
L-Glutamine	150	++	6.7
L-Alanine	82	++	6.2
L-Glutamic acid	105	++	7.0
L-Arginine[b]	100	++	6.5
L-Isoleucine	100	++	6.3
L-Serine	59	++	6.8
Glycine	32	+	6.6
Casamino Acids	350	+++	4.6
$NH_4 \cdot$ lactate (control)	100	++	7.2

[a] Ammonium lactate in the chemically defined medium was replaced as indicated. *S. kitasatoensis* KA-429 was cultivated for 5 days.
[b] Hydrochloride was used.

ence of more than 2 mM of ammonium ions, suggesting the operation of a nitrogen catabolite regulation.

It is believed that leucomycin is formed from four moieties, namely, the aglycone, mycarose, mycaminose, and side chains, which are synthesized by separate routes. The studies by Ōmura et al. of the ^{13}C precursors (8) showed that the aglycone moiety is synthesized from acetate, propionate, butyrate, and an undefined precursor via a "polyketide" intermediate. Sugar moieties (mycarose and mycaminose) may be synthesized from glucose, and leucine and valine were suggested to be the precursors of 4″ side chains (5). Accordingly, the biosynthesis of mycaminose and 4″ side chains, in which nitrogen metabolism is involved, is likely to be the target of the ammonia inhibition, but the effect on other components, cell wall and membrane transport, or on catabolism of leucomycin cannot be ruled out.

It is reasonable to suppose that the nitrogen catabolite regulation is reduced under a low ammonium ion concentration created by magnesium phosphate. Consequently, it may be concluded that magnesium phosphate caused a decrease of ammonium ions in the culture, which in turn resulted in high production of leucomycin. In support of this view, the use of a medium containing L-lysine or L-histidine as sole nitrogen source gave rise to higher production of leucomycin than did a medium containing ammonium ions (Table 2).

In view of the decline of the pH value of the magnesium phosphate-supplemented culture (Fig. 3), an alternative was considered that the stimulation might be a consequence of the pH effect, but the possibility was excluded because no significant increase of the leucomycin titer occurred under a condition of a constant pH value (S. Ōmura, Y. Tanaka, Y. Takahashi, H. Tanaka, and Y. Iwai, manuscript in preparation).

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