CALCIUM ION REGULATES AERIAL MYCELIUM FORMATION IN ACTINOMYCETES

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Ca^{2+} induced the formation of aerial mycelia in *Streptomyces ambofaciens*. Its effect was inhibited by ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, a Ca^{2+} specific chelating agent. A survey of 36 strains of actinomycetes showed that Ca^{2+} regulated aerial mycelium formation in 21 (58%) of them. The Ca^{2+} concentration required for aerial mycelium formation in the culture medium ranged from 0.1 to 1.5 mM.

Aerial mycelium formation and sporulation are two dramatic morphological events in the life cycle of actinomycetes. These cytodifferentiation processes have been found to be closely related to the production of secondary metabolites such as antibiotics.[1,2] We surveyed actinomycetes in which the formation of aerial mycelium was regulated by endogenous regulating substances and found several those produced aerial mycelium-inducing substances in the culture media. Of these, strains of *Streptomyces alboniger* and *Streptomyces ambofaciens* showed activity in a bioassay where their cultured agar discs were separated by a dialysis membrane from the test organisms. Thus the active substances were deduced to be of low molecular weight.

An aerial mycelium-inducing substance (pamamycin-607) was isolated from *S. alboniger* IFO 12738 by a bioassay that used an aerial mycelium-negative mutant of the same strain.[3] Pamamycin-607 is a novel, sixteen-membered macrodiolide with a dimethylamino group-bearing side chain.[4] At 0.1 μg/disc, pamamycin-607 induced aerial mycelia in the aerial mycelium-negative mutant.

S. ambofaciens KCC S-0204 formed abundant aerial mycelia on inorganic salts - starch (ISS) agar; but, when grown on yeast extract - malt extract agar (YMA), it produced only substrate mycelia; no aerial mycelium formed. Adding the broth from *S. ambofaciens* KCC S-0204 cultures grown in the ISS medium to substrate mycelia grown on YMA medium induced aerial mycelia. The active substance was purified by ion exchange and cellulose column chromatography, and identified as $Ca(OAc)_2$. It was assumed that the Ca^{2+} was derived from the $CaCO_3$ which was a component of ISS medium and had been solubilized as *S. ambofaciens* grew and acidified the culture medium.

Recently Ca^{2+} has been shown to have an important function as a second messenger in animals and plants.[5] We have investigated the aerial mycelium-inducing activity of Ca^{2+} in *S. ambofaciens* and we describe here its regulatory function in aerial mycelium formation by this and other actinomycetes.

Materials and Methods

Microorganisms and Chemicals

The 36 actinomycete species given in Table 2 were obtained from the Institute for Fermentation,

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Osaka and from Kaken Pharmaceutical Co., Ltd.

The $Ca(OAc)_2$, $CaCl_2$, $CaCO_3$ and $CoCl_2$ used were of special grade from Wako Pure Chemical Industries, Ltd. Ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was purchased from Dojindo Laboratories.

Yeast extract, malt extract and agar (1st grade) used for YMA medium were purchased from Oriental Yeast Co., Ltd., Difco Laboratories and Katayama Chemical Industries Co., Ltd., respectively.

Culture Media and Conditions

YMA medium[6] was used as the basal medium; its pH was adjusted to 7.3 before sterilization. A Petri dish (6-cm diameter) containing 7 ml of YMA medium was inoculated with actinomycete species and incubated at 28°C for 5 to 7 days.

Calcium salts ($Ca(OAc)_2$, $CaCl_2$ or $CaCO_3$) or EGTA was applied in two ways: 1) A paper disc was impregnated with a sterilized aqueous solution (pH of EGTA solution was adjusted to 7.3) and the paper disc was placed on the inoculated agar plate; 2) Ca^{2+} and/or EGTA was dissolved in the culture medium before pH adjustment. $CoCl_2$ was applied in the former way.

Quantification of Ca^{2+} in YMA Medium

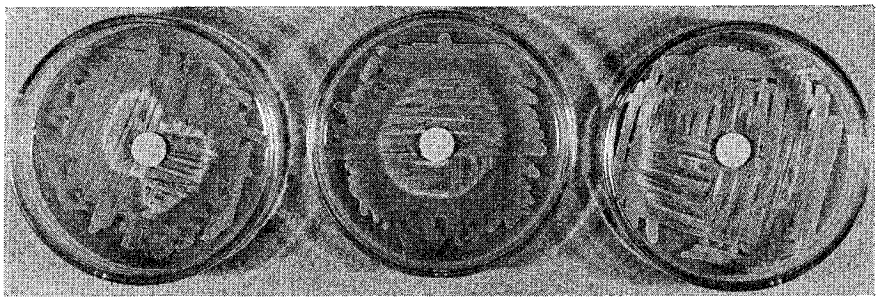
The Ca^{2+} concentration of the YMA medium was measured by atomic absorption spectrometry. YMA medium, which was prepared with 1 N HCl instead of distilled water, was filtered through Whatman No. 6 filter paper. To the filtrate, strontium chloride (1% w/v) was added to prevent interference by phosphate.[7] The Ca^{2+} concentration of the filtrate was measured with a Hitachi 170-50A atomic absorption spectrophotometer at 422.8 nm with an air-acetylene flame.

Results

Aerial Mycelium-inducing Activity of Ca^{2+} in *Streptomyces ambofaciens*

The aerial mycelium-inducing activity of $Ca(OAc)_2$ is shown in Fig. 1. At a dose of 2 μmol/disc, $Ca(OAc)_2$ produced a grayish zone of aerial mycelia, the diameter of which increased as the dose of $Ca(OAc)_2$ increased. We compared the activities of three Ca^{2+} salts (Table 1). The two water-

Fig. 1. Aerial mycelium-inducing activity of Ca^{2+} in *Streptomyces ambofaciens* KCC S-0204.



Ca^{2+} was applied as $Ca(OAc)_2$.

Table 1. Aerial mycelium-inducing activity of calcium salts in *Streptomyces ambofaciens*.

	1 (μmol/disc)	2 (μmol/disc)	5 (μmol/disc)	10 (μmol/disc)
$Ca(OAc)_2$	−	+	+	++
$CaCl_2$	−	+	+	++
$CaCO_3$	−	−	±	±

soluble salts, $Ca(OAc)_2$ and $CaCl_2$, had similar inducing activities, ranging from 2 to 10 μmol/disc, whereas, water-insoluble $CaCO_3$ showed no clear activity even at 10 μmol/disc. These results indicate that Ca^{2+} ion has aerial mycelium-inducing activity. Cultures incubated for more than a week with $CaCO_3$ began to show a narrow zone of aerial mycelium around the paper disc. This delayed activity of $CaCO_3$ is ascribed to its gradual solubilization as *S. ambofaciens* grew, presumably by excreting some acidic metabolites into the medium.

EGTA Inhibits the Aerial Mycelium-inducing Activity of Ca^{2+} in *S. ambofaciens*

To confirm that Ca^{2+} has aerial mycelium-inducing activity, we added EGTA, a Ca^{2+} specific chelating agent, to Ca^{2+}-containing media at various concentration ratios (Fig. 2). With smaller amounts of EGTA than Ca^{2+}, clear aerial mycelium formation was observed. At equimolar concentrations of Ca^{2+} and EGTA, only sparse aerial mycelia formed. When EGTA exceeded Ca^{2+}, aerial mycelium formation was inhibited but substrate mycelia grew well. We concluded that Ca^{2+} is necessary for aerial mycelium formation by *S. ambofaciens* KCC S-0204.

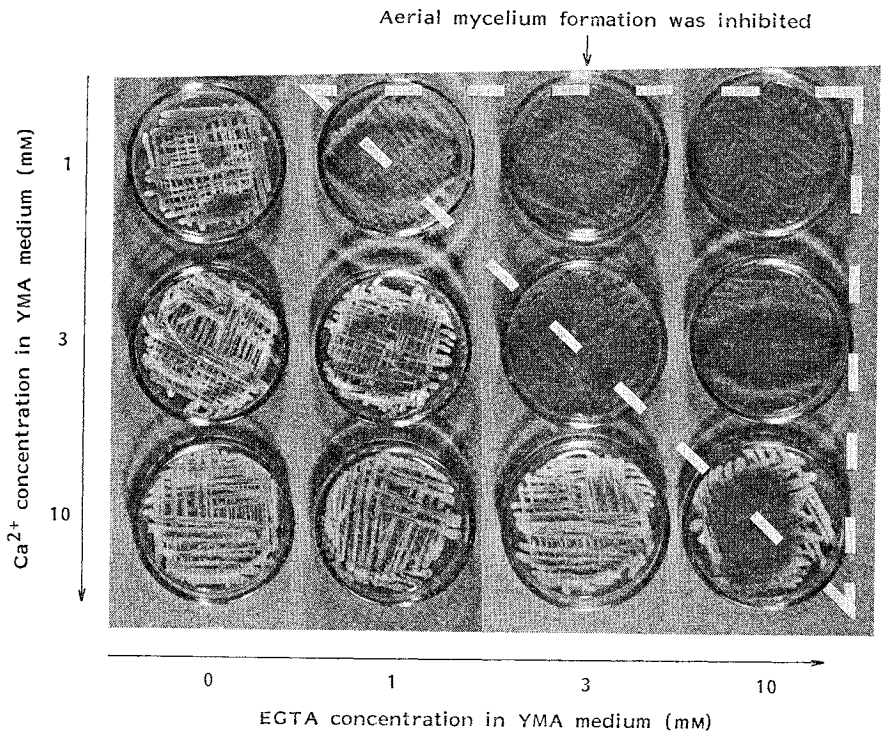
Effects of Ca^{2+} and EGTA on Various Actinomycete Species

We assayed the effect of Ca^{2+} and EGTA on aerial mycelium formation by 35 actinomycete species. $CaCl_2$ (10 mM) was dissolved in YMA medium. EGTA was applied to the paper disc at a dose of 3 μmol/disc. Results showed the actinomycete species to be of four types (A~D) (Table 2), typical examples of which are shown in Fig. 3.

Type A

Strains in which aerial mycelium formation was induced by Ca^{2+}.

Fig. 2. EGTA inhibition of the aerial mycelium-inducing activity of Ca^{2+} in *Streptomyces ambofaciens*.



In addition to *S. ambofaciens* KCC S-0204, this group contained *S. alboniger* IFO 12738. When Ca^{2+} was added to the medium, the smooth ocherous colonies of *S. alboniger* became powdery white. This Ca^{2+}-induced aerial mycelium formation was inhibited by EGTA as with *S. ambofaciens*.

Type B

Strains in which aerial mycelium formation was stimulated by Ca^{2+}.

Table 2. Effects of Ca^{2+} and EGTA on various actinomycete species.

Type	Strain	Aerial mycelium formation		
		YMA	Induction or stimulation by Ca^{2+} (10 mM)	Inhibition by EGTA (3 μmol/disc)
Type A: Strains in which aerial mycelium formation was induced by Ca^{2+}				
	Streptomyces alboniger IFO 12738	−	+	(+)[a]
	S. ambofaciens KCC S-0204	−	+	
Type B: Strains in which aerial mycelium formation was stimulated by Ca^{2+}				
	Streptomyces griseospiralis KCC S-0869	+	+	+
	S. hygroscopicus KCC S-0439	+	+	+
	S. kitasatoensis KCC S-1001	+	+	+
	S. olivaceus IFO 12805	+	+	+
	S. tendae KCC S-0149	+	+	+
Type C: Strains in which aerial mycelium formation was inhibited by EGTA				
	Streptomyces albidoflavus IFO 13010	+	−	+
	S. argenteolus KCC S-0229	+	−	+
	S. fradiae IFO 12773	+	−	+
	S. fulvoviridis KCC S-0374	+	−	+
	S. griseus IFO 3102	+	−	+
	S. janthinus IFO 12879	+	−	+
	S. kanamyceticus IFO 13414	+	−	+
	S. kitasatoensis KCC S-1000	+	−	+
	S. lavendulae IFO 12789	+	−	+
	S. lipmanii IFO 12791	+	−	+
	S. rimosus IFO 3228	+	−	+
	Saccharopolyspora erythraea IFO 13426	+	−	+
	Streptoverticillium albireticuli KCC S-0116	+	−	+
	S. griseocarneum IFO 3387	+	−	+
Type D: Strains in which aerial mycelium formation was not affected by addition of Ca^{2+} or EGTA				
	Streptomyces humifer IFO 13342	−	−	
	S. glomeroaurantiacus IFO 13380	−	−	
	S. horton IFO 13355	−	−	
	S. lactamdurans IFO 13305	−	−	
	S. luteolutescens IFO 13489	−	−	
	S. olivochromogenes IFO 13067	−	−	
	S. sclerotialus IFO 13356	−	−	
	S. violens IFO 13486	−	−	
	S. aureofaciens IFO 12843	+	−	−
	S. cattleya KCC S-0925	+	−	−
	S. cirratus KCC S-0738	+	−	−
	S. coelicolor IFO 3176	+	−	−
	S. eurythermus KCC S-0206	+	−	−
	S. flavus KCC S-0036	+	−	−
	S. kasugaensis IFO 13851	+	−	−
Number of strains		36	7 (19%)	21 (58%)

[a] EGTA inhibited the aerial mycelium formation induced by Ca^{2+} (see Fig. 2 and text).

Table 3. Recovery of EGTA inhibition of aerial mycelium formation by excess Ca^{2+}.

Strains	Aerial mycelium formation		
	YMA	1 mM EGTA	1 mM EGTA, 5 mM $CaCl_2$
Streptomyces albidoflavus IFO 13010	+	−	+
S. argenteolus KCC S-0229	+	±	+
S. fradiae IFO 12773	+	−	+
S. fulvoviridis KCC S-0374	+	−	+
S. griseus IFO 3102	+	±	+
S. janthinus IFO 12879	+	±	+
S. kanamyceticus IFO 13414	+	−	+
S. kitasatoensis KCC S-1000	+	±	+
S. lavendulae IFO 12789	+	−	+
S. lipmanii IFO 12791	+	−	+
S. rimosus IFO 3228	+	−	+
Saccharopolyspora erythraea IFO 13426	+	−	+
Streptoverticillium albireticuli KCC S-0116	+	±	+

There were 5 strains in this group. Without addition of Ca^{2+}, they produced aerial mycelia sparsely on YMA medium; addition of Ca^{2+} stimulated aerial mycelium formation. *Streptomyces hygroscopicus* KCC S-0439 and *Streptomyces kitasatoensis* KCC S-1001 showed the most marked morphological changes (Fig. 3).

As additional evidence that aerial mycelium formation by type B strains is regulated by Ca^{2+}, EGTA caused inhibition.

Type C

Strains in which aerial mycelium formation was inhibited by EGTA.

Strains in this group formed abundant aerial mycelia when grown on YMA medium. Addition of Ca^{2+} had no effect on the appearance of the colonies. However, when EGTA was applied in paper discs, a definite aerial mycelium-inhibitory zone was visible around disc, whereas, substrate mycelia grew well.

Evidence that the inhibition of aerial mycelium formation by EGTA on these type C strains is the result of Ca^{2+}-starvation was obtained as follows (Table 3): EGTA, added to the medium at 1 mM inhibited or markedly diminished production of aerial mycelia. This inhibition was removed by excess (5 mM) Ca^{2+}. We concluded that aerial mycelium formation by type C strains also is regulated by Ca^{2+}, bringing the total to 21 (types A, B and C) (58%) of the 36 strains tested.

Type D

Other strains. The other 15 strains tested were not induced to form aerial mycelium by Ca^{2+} nor inhibited by EGTA. Of these, 8 strains formed no aerial mycelium on YMA, ISS, BENNETT's or ATCC sporulation agar media. If we regard these 8 strains as essentially lacking the ability to form aerial mycelium, the percentage of the sporulating strains affected by Ca^{2+} or EGTA increases to 75%.

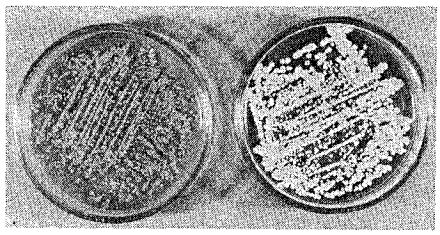
Ca^{2+} Concentration Required for Aerial Mycelium Formation

Type A strains should require much more Ca^{2+} than the other types, because they needed extra Ca^{2+} in addition to that contained in YMA medium. *S. alboniger* IFO 12738 and *S. ambofaciens* KCC S-0204 were cultured on YMA media supplemented with $CaCl_2$ at concentrations of 0.3, 1.0

Fig. 3. Effects of Ca^{2+} and EGTA on various actinomycetes.

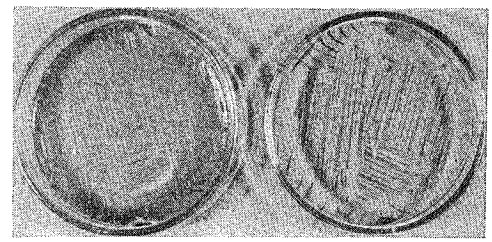
Actinomycetes could be classified into four types (the strain type shown in Table 2).

(A) *Streptomyces alboniger* IFO 12738 (type A)



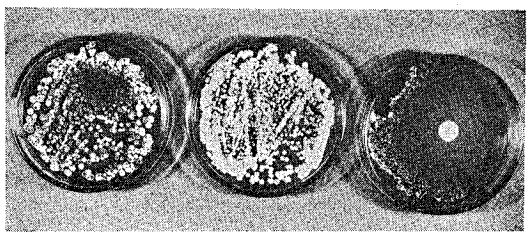
YMA medium + $CaCl_2$ (10 mM)

(B) *Streptomyces ambofaciens* KCC S-0204 (type A)



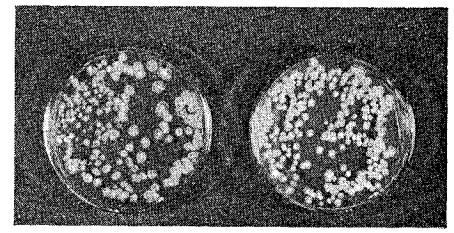
YMA medium + $CaCl_2$ (10 mM)

(C) *Streptomyces hygroscopicus* KCC S-0439 (type B)



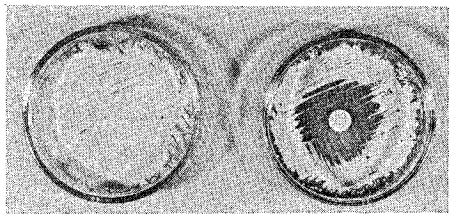
YMA medium + $CaCl_2$ (10 mM) EGTA (3 μmol/disc)

(D) *Streptomyces kitasatoensis* KCC S-1001 (type B)



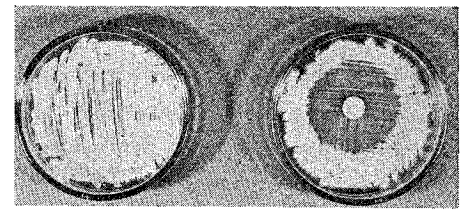
YMA medium + $CaCl_2$ (10 mM)

(E) *Streptomyces lavendulae* IFO 12789 (type C)



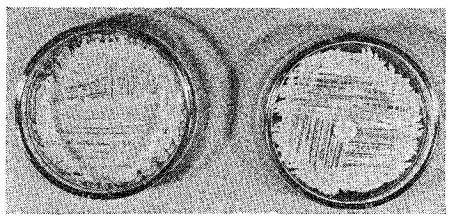
YMA medium EGTA (3 μmol/disc)

(F) *Streptomyces lipmanii* IFO 12791 (type C)



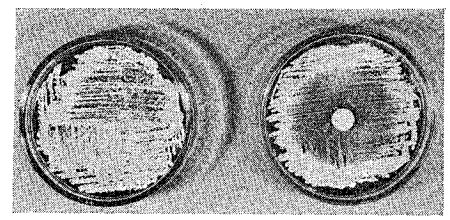
YMA medium EGTA (3 μmol/disc)

(G) *Streptomyces janthinus* IFO 12879 (type C)



YMA medium EGTA (3 μmol/disc)

(H) *Saccharopolyspora erythraea* IFO 13426 (type C)



YMA medium EGTA (3 μmol/disc)

and 3.0 mM. At 1.0 mM Ca^{2+}, *S. alboniger* formed aerial mycelia; it required the highest concentration of Ca^{2+} among the actinomycetes tested (Table 4). The Ca^{2+} content of YMA medium itself was determined to be 0.5 mM by atomic absorption analysis. Thus, 1.5 mM of Ca^{2+} in the medium

Table 4. Ca^{2+} concentrations required for aerial mycelium formation by actinomycetes.

Type	Strains	Concentration of $CaCl_2$ (mM)		
		0.3	1.0	3.0
Type A: Induction by Ca^{2+}				
	Streptomyces alboniger IFO 12738	−	+	++
	S. ambofaciens KCC S-0204	±	+	++
		Concentration of EGTA (mM)		
		0.2	0.4	0.8
Type C: Inhibition by EGTA				
	Streptomyces fradiae IFO 12773	++	+	−
	S. fulvoviridis KCC S-0374	++	+	−
	S. lavendulae IFO 12789	++	++	−
	S. lipmanii IFO 12791	++	++	−
	Saccharopolyspora erythraea IFO 13426	±	−	−

Ca^{2+} concentration in YMA medium was determined to be 0.5 mM by atomic absorption analysis.

was required for aerial mycelium formation by *S. alboniger* IFO 12738.

Since strains of type C formed abundant aerial mycelia on YMA medium, the Ca^{2+} present must be sufficient for them. We attempted to immobilize this Ca^{2+} by adding EGTA at concentrations of 0.2, 0.4 and 0.8 mM. Even in the presence of 0.4 mM EGTA, aerial mycelium formation by *Streptomyces lavendulae* IFO 12789 and *Streptomyces lipmanii* IFO 12791 was not inhibited. This indicates that 0.1 mM of Ca^{2+} is sufficient for aerial mycelium formation by these strains.

Thus, the concentration of Ca^{2+} in the medium required for aerial mycelium formation ranged from 0.1 to 1.5 mM.

Effect of Co^{2+} on Actinomycete Species

We also examined the effect of Co^{2+} on aerial mycelium formation by the paper disc method. At 1 and 5 μmol/disc, Co^{2+} induced aerial mycelium formation by *S. ambofaciens* KCC S-0204 and stimulated that by *S. kitasatoensis* KCC S-1001, and it also restored aerial mycelium formation after EGTA-inhibition (1 mM) in 5 of the type C strains. The definite difference between Co^{2+} and Ca^{2+} was that Co^{2+} caused growth inhibition around the paper disc at a dose required for aerial mycelium formation. At 1 μmol/disc, *S. ambofaciens* formed a growth inhibitory zone (1.6-cm diameter) around the paper disc, the outer area of which was an aerial mycelium-inducing zone (0.8 cm in width). At 5 μmol/disc, Co^{2+} caused growth inhibition around the disc in all the strains tested.

Discussion

Cytodifferentiation of actinomycetes is regulated by endogenous substances such as A-factor[8] and factor-C[9]. McCann and Pogell showed that *S. alboniger* produced the regulating substance, pamamycin, which they obtained as a mixture of four homologues (MWs 621, 635, 649 and 663).[10] We isolated a new pamamycin homologue, pamamycin-607 (MW 607) in a pure form and elucidated its structure.[3,4]

In the culture media used for growth of actinomycetes, $CaCO_3$ is frequently one of the components. Its role hitherto was considered to be maintaining neutral pH conditions. Our present research, however, has shown that $CaCO_3$ has another function; induction or stimulation of cytodifferentiation such as aerial mycelium formation.

Hickey and Tresner reported that Co^{2+} improved the rate and extent of sporulation of *Streptomyces* species.[11] We examined the effect of Co^{2+} on aerial mycelium formation. Aerial mycelium

formation of 7 strains was effected by Co^{2+}, but growth of all the strains tested was inhibited at a same dose required for aerial mycelium formation. This result may indicate that aerial mycelium formation by Co^{2+} is an indirect effect of growth inhibition.

Recently the intracellular Ca^{2+} concentration of *Escherichia coli* was measured by GANGOLA and ROSEN[12]; when the Ca^{2+} concentration in the medium was increased from 0.08 to 10 mM, the concentration of free Ca^{2+} in the cells was maintained at 0.1 μM, although the total amount of Ca^{2+} increased in parallel with the extracellular Ca^{2+}. With actinomycetes we have now shown that the extracellular Ca^{2+} concentration required for aerial mycelium formation ranges from 0.1 to 1.5 mM. This is within the range of Ca^{2+} in the medium used for *E. coli* by GANGOLA and ROSEN. We suggest that the free Ca^{2+} concentration in actinomycetes cells is probably comparable to that in *E. coli* cells.

From its macrodiolide structure, we expected that pamamycin-607 might be a Ca^{2+} transporter. However, this could not be demonstrated by *in vitro* two phase partition experiments.[8] Examination of CPK space filling atomic models indicates that the sixteen-membered macrodiolide ring of pamamycin-607 has insufficient space to incorporate Ca^{2+}. However, we found that pamamycin-607 has anion transporting ability; in its presence MnO_4^- and Cl^- transfer from water to the benzene layer.[3] CHOW and POGELL reported that one of the primary target of pamamycins in growth inhibition of bacteria was inhibition of phosphate transport.[13] As one possible explanation why pamamycin-607 and Ca^{2+} exhibit similar activity in *S. alboniger*, Ca^{2+} might lessen the intracellular concentration of phosphate by forming insoluble calcium phosphate; sequestration of phosphate in the cells could be related to aerial mycelium formation.

Since Ca^{2+} showed activity in a wide range of actinomycete species, the mode of action of Ca^{2+} in aerial mycelium formation is of great interest.

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