CHARACTERIZATION OF AN INDUCIBLE TRANSPORT SYSTEM FOR GLYCEROL IN *Streptomyces clavuligerus*

REPRESSION BY L-SERINE

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Streptomyces clavuligerus NRRL 3585 grown in a chemically defined medium containing glycerol as the sole carbon source transported this molecule by two different systems. One of these was constitutive with a very low uptake efficiency and insufficient to attend to the metabolic requirements of this bacterium (constitutive glycerol transport system) and the other (glycerol transport system (GTS)) active and specifically induced by D-glycerol which is responsible for the transport of more than 90% of the glycerol taken up the cells. GTS was seen to have an optimal pH and temperature of 7.0 and 30°C, respectively, and its *Km* was 14 μM. It was repressed by L-serine and addition of this amino acid to the culture broth (10 mM) inhibited the growth of *S. clavuligerus* but not that of other species of *Streptomyces*.

It is well known that the utilization of different carbon sources by *Streptomyces* species is very broad[1~3]. However, *Streptomyces clavuligerus* is an exception among them, since this species has important metabolic limitations. Thus, this bacterium grows efficiently in culture media containing glycerol or maltose as the sole carbon sources, whereas in those containing other sugars or complex carbohydrates it either does not grow or does so very poorly (dextrin and starch)[2]. Despite this, *S. clavuligerus* is a very important industrial species since it produces several β-lactam antibiotics, including the potent β-lactamase inhibitor clavulanic acid[4]. The clinical importance of this compound led many scientists to study its biosynthetic pathway in an attempt to establish the regulatory mechanisms controlling the production of this molecule[5~10]. Although in recent years some aspects have been approached[11~12], the main enzymatic steps and the nature of some of the biosynthetic intermediates remain almost unknown. SALOWEE *et al.*[13] have recently shown that D-glyceric acid, a derivative of glycerol, is a direct precursor of clavulanic acid, thus highlighting the importance of glycerol in the biosynthesis of this antibiotic. It therefore seems evident that the characterization of the glycerol transport system (GTS), knowledge of its optimal physico-chemical parameters and their effectors should have important industrial implications; this is the main objective of this work.

Materials and Methods

Chemicals

3-(*N*-Morpholino)propanesulfonic acid (MOPS) was from Sigma Chemical Co. (St. Louis, Mo. U.S.A.). [*U*-^{14}C]Glycerol (165 mCi/mmol) was purchased from Amersham (UK). L-[^{14}C]Serine (179 mCi/mmol) was from New England Nuclear (UK). All other chemicals used were of analytical quality.

Microorganisms

Streptomyces clavuligerus NRRL-3585; *Streptomyces lividans* ATCC 19844; *Streptomyces acrymicini* ATCC 19885 and *Streptomyces griseus* IMRU-3570 were obtained from the corresponding culture collections. Spores of the different strains were obtained as reported[14] and kept frozen at −20°C in 20% (w/v) glycerol[15].

Culture Media and Growth Conditions

The medium used for the growth of *S. clavuligerus* was a chemically defined one[16] (MG) with the following composition (g/liter): D-glycerol, 10; NH_4Cl, 2.14; $MgSO_4 \cdot 7H_2O$, 0.6; HK_2PO_4, 4.4; $FeSO_4 \cdot 7H_2O$, 0.001; $MnCl_2 \cdot 4H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; $CaCl_2 \cdot 2H_2O$, 0.001; MOPS, 21; distilled water to 1 liter. The medium was adjusted to pH 7.0 with 1 M NaOH. When required, D-glycerol was replaced by maltose (MM), keeping the final concentration constant. Each 250 triple baffled Erlenmeyer flask containing 50 ml of the MG (or MM) medium was inoculated with 0.2 ml of the spore suspension (10^8 spores/ml) and incubated on a rotary shaker (250 rpm) at 30°C for the time required in each set of experiments. Glycerol, added as a protectant to the spore suspension, was removed by washing spores three times with sterile distilled water.

When solid media were required, agar (20 g/liter) was added. To study the effect of L-serine on the growth of different *Streptomyces* species, each plate, containing 20 ml of freshly prepared MG, was supplied with 2 ml of a 110 mM solution of L-serine (final concentration 10 mM).

Uptake of [*U*-^{14}C]Glycerol

Cells grown as above were harvested at different times by filtration on Whatman No. 1 filter paper and washed three times with sterile saline. Cells were resuspended in MG without glycerol and adjusted to an A_{600} of 0.5 (1 ml of cells with an A_{600} of 0.5 contains to 0.18 mg dry weight of cells). Aliquots of 1 ml were placed in 25-ml Erlenmeyer flasks and preincubated at 30°C for 4 minutes in a thermostatically controlled water bath (160 strokes/minute) before adding glycerol (25 μM or other concentration, containing 0.55 μM [*U*-^{14}C]glycerol). Incubations were carried out for 1 minute unless stated otherwise. Uptake was halted by adding 10 vol of a solution of glycerol (50 mM). Cells were rapidly filtered through Millipore filters (0.45 μm pore size) and washed with 3 × 10 ml of sterile distilled water. Filters were dissolved in 10 ml of scintillation fluid and counted as reported[17,18].

When required, potential effectors (5 mM) were added to the uptake mixture 2 minutes before the [*U*-^{14}C]glycerol. To study the effect of arsenate, phosphate was eliminated. In these cases the concentration of arsenate tested ranged between 5 ~ 50 mM.

[^{14}C]Glycerol uptake is given as nmol/minute/ml cells of $A_{600}=0.5$ or as nmol/ml cells of $A_{600}=0.5$ when different uptake periods were employed.

Uptake of L-Serine

L-[*U*-^{14}C]Serine (179 mCi/mmol) was used to study the uptake of this amino acid. Uptake rates were followed under similar conditions to the above but in this case only labeled L-serine (0.5 μM) was used.

Half-life Calculation of GTS

To determine the half-life of the GTS in *S. clavuligerus*, cells, grown in MG for 85 hours, were collected, washed three times with sterille saline and resuspended in freshly prepared MG (A_{600} 1.0). To each Erlenmeyer flask, 100 μg of chloramphenicol was added per ml and the glycerol transport rate was studied at different times of incubation.

Induction Experiments

Cells grown in MM as above were harvested at 96 hours, washed and suspended in MG without glycerol (A_{600} 1.0). Each 250 triple baffled Erlenmeyer flask, containing 50 ml of this medium, was incubated at 30°C on a rotary shaker (250 rpm) for different times (0 ~ 6 hours). Induction of GTS was started by adding glycerol (0.1% w/v). Later, cells were harvested at different times and the rate of uptake was analyzed as indicated above. To study the effect of different molecules on the induction of GTS, they were added to the flasks at induction time (final concentration 0.1%, w/v), unless otherwise stated.

Results and Discussion

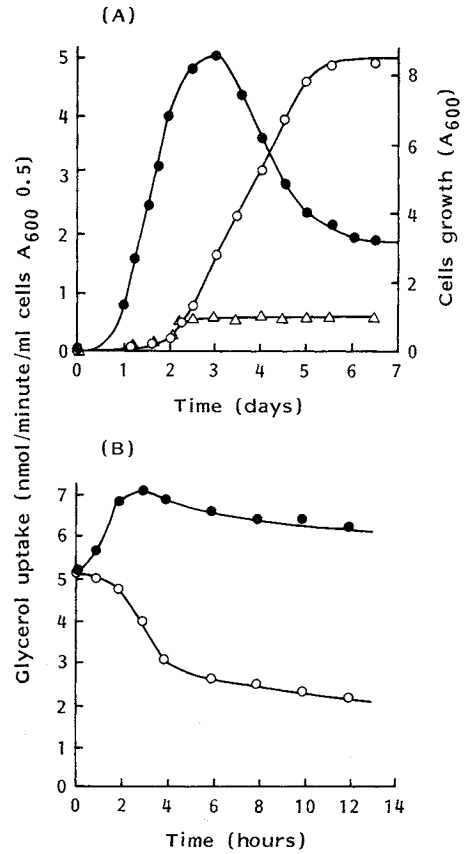
Time Course of the Appearance of GTS in *S. clavuligerus*

The appearance of the glycerol transport system (GTS) in *S. clavuligerus* was studied by growing this

bacterium in a chemically defined medium containing maltose (MM) or glycerol (MG) as the sole carbon sources. In both media (MM and MG), the growth was similar; however, in the first case the transport rate was very low remaining constant from 48 to 144 hours, (constitutive glycerol transport system, CTS, see Fig. 1A) whereas in the second one *S. clavuligerus* showed a variable capacity of transport from 0 to 144 hours (Fig. 1A). Thus, in the first hours of growth (0 ~ 10 hours), corresponding to the time of spore germination, transport was very low or nil, whereas from 12 to 72 hours it increased continuously. From 72 hours until the late logarithmic phase of growth the uptake of glycerol decreased, remaining constant during the stationary phase. These results indicated that the main GTS, responsible for the uptake of more than 90% of the glycerol, is an inducible system and therefore mediated by a protein(s) not present in cells grown in media without glycerol (MM). The half-life of GTS (about 8 hours) (Fig. 1B) was calculated by adding 100 μg/ml of chloramphenicol to 85 hours old cultures (see Materials and Methods). The half-life value estimated is in full agreement with the other ones reported for other bacterial or fungal permeases[18,19].

Fig. 1. Glycerol uptake in *Streptomyces clavuligerus* grown in a minimal medium containing glycerol (MG).

(A) Time course of the appearance of GTS (●) and cellular growth (○) of *S. clavuligerus* NRRL-3585 grown in MG. Transport of [*U*-^{14}C]glycerol when cells were grown in MM (△). (B) Half-life determination of GTS. Cells incubated in the presence (○) or absence (●) of chloramphenicol (100 μg/ml).



Characteristics of GTS

The optimal temperature for GTS ranges

Fig. 2. Effect of (A) temperature; (B) pH (100 mM citrate phosphate buffer, △; 100 mM phosphate, □; MG without glycerol adjusted at different pH values, ●; 100 mM HCl-Tris, ○) and (C) time on GTS.

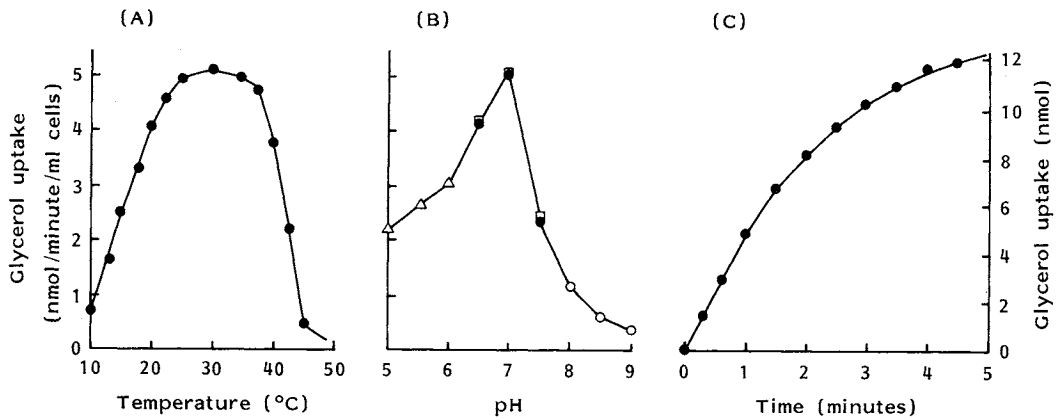


Fig. 3. Kinetics of GTS. Plot of 1/V *versus* 1/[S].

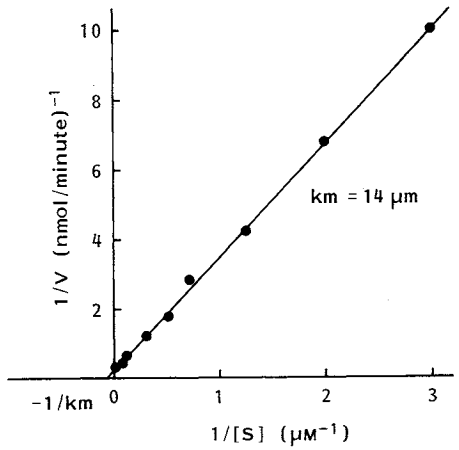


Table 1. Influence of different ions, thiol-containing and thiol-modifying reagents on GTS.

Effectors (5 mM)	Inhibition (%)
Hg^{2+}	99
Cu^{2+}	82
Fe^{2+}	70
N-Ethylmaleimide	60
DTNB	62
Iodoacetate	75

Table 2. Effect of acetic acid and different fatty acids on GTS.

Compounds (5 mM)	Inhibition (%)
Acetic acid	0
Propionic acid	0
Butyric acid	0
Pentanoic acid	0
Hexanoic acid	3
Heptanoic acid	12
Octanoic acid	66
Nonanoic acid	79
Decanoic acid	99

between 23 and 35°C (Fig. 2A). Glycerol uptake was maximal at pH 7.0 (Fig. 2B). A similar optimal pH value has been reported for GTS in *Streptomyces coelicolor*[20]. Under the above conditions, GTS was a linear function of time for at least 90 seconds although to carry out the assays under good conditions of linearity, 60 seconds periods of uptake were routinely employed (Fig. 2C). Under these assay conditions, the calculated *Km* for the GTS was 14 μM (Fig. 3), suggesting that in *S. clavuligerus* the permease for glycerol has a high affinity for the substrate. This result contrasts with the higher *Km* values reported for other permeases from *Streptomyces* involved in the uptake of different carbon sources[20~22].

The specificity of the GTS was examined by adding different molecules to the uptake mixture. When carbohydrates (xylose, ribose, L-arabinose, galactose, mannose, glucose, fructose, glucosamine, galactosamine, mannosamine, maltose, lactose, sucrose and trehalose); amino acids; metabolic intermediates (lactate, pyruvate, 3-phosphoglycerate, phosphoenolpyruvate, citrate, fumarate and 2-oxoglutarate), and sugar derivatives (*N*-acetylglucosamine, *N*-acetylmannosamine) or other molecules (mannitol, sorbitol, ethyleneglycol and polyethyleneglycol) were tested, almost all of them failed to cause any effect. Only the related structural compound, ethyleneglycol (40 mM), led to inhibition (about 60%). These results suggest that, in *S. clavuligerus*, the inducible GTS is a very specific transport system.

Influence of Different Ions and Thiol-containing or Thiol-modifying Reagents

GTS was strongly inhibited by certain divalent cations (Hg^{2+}, Cu^{2+} and Fe^{2+}) and also by the thiol-modifying reagents *N*-ethylmaleimide, DTNB and iodoacetate (Table 1) suggesting the existence of an essential SH group in the permease[19,23,24]. Other ions (Na^+, K^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+} and Mn^{2+}) and thiol- containing compounds (β-mercaptoethanol, cysteine, cystine, cysteamine and homocysteine) did not cause any effect.

Effect of Fatty Acids

Some fatty acids are able to affect the transport rates of many molecules through biological membranes and also the activity of enzymes (or enzymatic systems) associated with or included in them[18,25]. To examine the effect of these compounds on GTS, acetic acid and several fatty acids were added to the assay

system (Table 2). Only fatty acids whose carbon chain length between C_6 to C_{10} atoms caused inhibition, which increased the total length of the acyl-chain increased. This effect, which can be explained by the detergent properties of the active compounds[18], suggests that intact membranes are needed to maintain a functional transport system[18,25].

Effect of Metabolic Inhibitors

KCN inhibited GTS in *S. clavuligerus* by more than 80% whereas arsenate only poorly affected the system (20%). Moreover, the energy poisons 2,4-dinitrophenol (2,4-DNP) and 4-nitrophenol (4-NP) also inhibited uptake (84 and 81%, respectively) suggesting that GTS is an energy dependent transport system and that the energy production might be intimately coupled to transport within the cell membrane. Similar results have been reported for the GTS in *S. coelicolor*[1]. The poor effect of arsenate (even when added at 50 mM) indicates that ATP is not the energy source for this active mechanism[17,18,26].

It is surprising that an active transport system involved in the uptake of glycerol exists in *S. clavuligerus* since it is a rate event in nature which has only been reported in yeast, some molds and algae. These organisms have low permeability to glycerol in contrast to bacteria which are quite permeable to glycerol[27~31]. For these reasons, it could be speculated that results above did not represent a true measure of active transport but rather an indirect measure of some of the enzymes involved in the catabolism of this compound. It has been reported that in *S. coelicolor* two different enzymes, ATP-dependent glycerol kinase and a NAD-independent glycerol-3-phosphate dehydrogenase are co-ordinately induced by addition of glycerol to cultures growing in media containing other carbon sources[20]. However, experimental data allowed us to rule out this possibility as follow:

a) If the data showed correspond to the kinetic measurement of the first enzyme (ATP-dependent glycerol kinase), how can the poor effect of arsenate be explained? It could be considered that the ATP-pool was not exhausted after adding arsenate (2 minutes before glycerol, see Materials and Methods), but if this were the case, how can one explain that KCN, added at the same time, blocks the uptake by more than 80%? Furthermore, long-chain fatty acids strongly inhibit the uptake of glycerol suggesting that disorganization of the cellular membrane, due to their detergent properties[18,25] occurs. However, glycerol kinase should not be affected, at least significantly, since is a soluble enzyme[20].

b) The low effect of arsenate and the strong inhibition caused by some fatty acids could be explained if we are measuring the second enzyme (NAD-independent glycerol-3-phosphate dehydrogenase) which is a membrane-associated protein. However, in this case, how can one explain the inhibition by KCN? This metabolic inhibitor is usually added (1 mM) to the reaction mixtures employed for the assay of this enzyme (see ref 20). Moreover, the *Km* of this enzyme is 7 mM[20], about 500 times higher than the *Km* calculated for the transport system reported here (14 μM). For all these reasons we conclude that there exists an active glycerol transport system in *S. clavuligerus*. Similar results have also been reported by other authors studying the glycerol transport system in *S. coelicolor*[1] and in a mutant of *S. clavuligerus*[32].

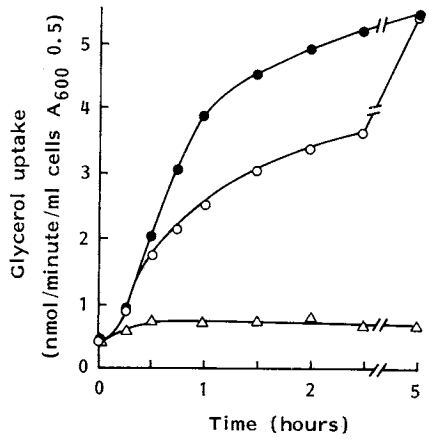
Induction of GTS

Control by induction-repression of the transport systems is a well-known genetic mechanism that finely regulates the quantity of permease present in the cell membranes[33]. We have indicated above that GTS, in *S. clavuligerus*, is an inducible transport system which only appeared when this bacterium was grown in a medium containing glycerol as the carbon source (Fig. 1). To study the induction of GTS, cells grown in maltose were harvested, washed and resuspended in the same medium containing glycerol.

The GTS appeared rapidly (indeed after 20 minutes) and increased continuously for 2 hours; later it remained almost constant (Fig. 4). However, when cells grown under similar conditions were transferred to maltose medium, GTS induction was not observed. Furthermore, in the cases in which cells were resuspended in minimal medium containing maltose plus glycerol (MMG), the induction rate was slower during the first hours but, later (after 5 hours), the same transport capacity was observed (Fig. 4). This delay can be explained as follows, *S. clavuligerus*, when previously grown in MM, has all the enzymes necessary for the catabolism of maltose and therefore does not require glycerol uptake for growth until disappearance of maltose.

The influence of different potential effectors (sugars, amino acids, or derivatives) on the induction of GTS was studied by supplying these compounds to the induction medium. None of the sugars tested as well as certain of the amino acids (Ala, Thr, Met, Arg, His, Lys, Orn, Pro, Trp, Phe, Tyr and homoserine) had any effect; whereas others (Gly, Val, Leu, Ile, Glu, Asp, Gln and Asn) slowly increased induction (between 10~15%). However, L-cysteine, L-serine and certain structural analogues (ethanolamine, cysteamine, β-mercaptoethanol) strongly repressed the induction of GTS (94, 73, 45, 54, 49%, respectively); whereas alanine, ethylamine, homoserine, homocysteine and threonine did not affect it or very poorly (<5%).

Fig. 4. Induction of GTS by glycerol when *Streptomyces clavuligerus* was grown in minimal medium containing glycerol (●), maltose (△), or maltose and glycerol (○).



Structural Hypothesis

To further characterize this repression L-serine, but not L-cysteine, was used since the latter molecule could cause non-specific effects on many different enzymes (or enzymatic systems) that might indirectly affect the synthesis of the protein(s) involved in GTS.

When different concentrations of L-serine were tested (Fig. 5A), we found that even at very low

Fig. 5. Effect of different concentrations (0.1, □; 0.5, ○; 1, ▲; 5, △ and 10 mM, ■) of L-serine (A) and its addition (10 mM) at different times (B) on the induction of GTS. Control without serine (●). Control without glycerol (non-induced cells) (▽).

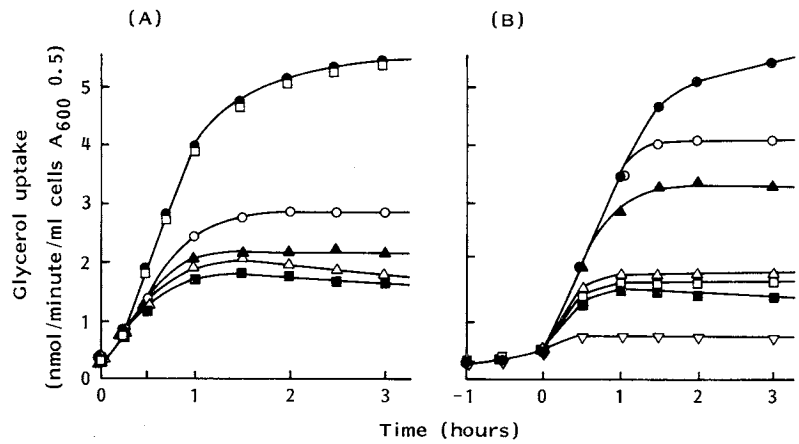


Fig. 6. Transport of L-serine by *Streptomyces clavuligerus* grown in MM.

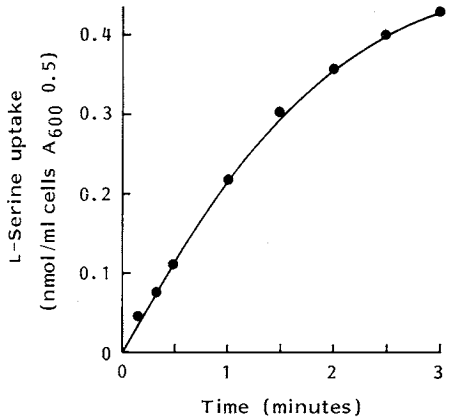
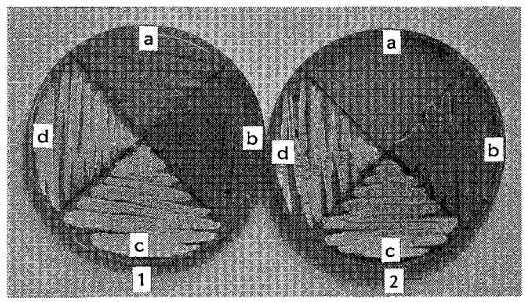


Fig. 7. Effect of L-serine (10 mM) on the growth of different species of *Streptomyces* (*S. clavuligerus*, a; *S. lividans*, b; *S. griseus*, c; *S. acremycini*, d). 1, MG without L-serine and 2, MG supplied with the amino acid.



concentrations (0.5 mM), repression occurred and that the addition of this amino acid to the cultures at different induction times (or before induction) required a certain amount of time (about 30 minutes) to cause the effect (Fig. 5B). These last results can be explained either by the absence of permeases for L-serine, that would be induced, or by the slowness of the repression process, which probably requires the transformation of L-serine into some catabolic intermediate. This latter assumption seems to be the case, since *S. clavuligerus* has a constitutive transport system for L-serine (Fig. 6).

If GTS is in fact repressed by L-serine, it seemed reasonable to suppose that supplementation of the MG medium with L-serine should prevent the growth of *S. clavuligerus*. To test such a hypothesis, spores of *S. clavuligerus* were inoculated on solid MG medium with or without L-serine (10 mM). Figure 7 shows that L-serine strongly inhibited the growth of this bacterium and that this effect is specifically caused in *S. clavuligerus* since other species of *Streptomyces* (*S. lividans*, *S. griseus* and *S. acrimycini*) were unaffected.

The absence of growth in solid medium (Fig. 7) also allows us to conclude that, in *S. clavuligerus*, the non-inducible glycerol transport system (CTS), which is not repressed by L-serine (see Fig. 5B), is insufficient to transport the glycerol required for supporting bacterial growth.

Apart from establishing an additional difference between *S. clavuligerus* and other species of the genus, all the foregoing results might have important industrial implications. Thus, most of the fermentation media used for clavulanic acid production contain glycerol and different protein-rich raw materials to be used by *S. clavuligerus* as carbon and nitrogen sources. Therefore, an unusually high concentration of L-serine in them could dramatically affect the growth of this bacterium and hence the final titer of antibiotic. We suggest that strict control over the free levels of L-serine and L-cysteine in the raw materials would be a good strategy to assure high production of clavulanic acid in industrial fermentations.

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