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Intracellular acetyl-CoA depletion by the cat gene is responsible for growth inhibition of Escherichia coli C584 on M9 minimal media

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Two closely related strains of Escherichia coli, C584 and DH5α, differ in their ability to grow on M9 minimal media supplemented with glycerol and thiamine. While the DH5α strain is able to grow in the nutrient-limited medium, the identical C584 strain containing the pMOB3 plasmid fails to do so. The chloramphenicol resistance cat gene encoded on the pMOB3 plasmid has been implicated in this growth inhibition. The proposed mechanism of the inhibition is by the action of the cat gene product chloramphenicol acetyl transferase (CAT). CAT utilizes acetyl-CoA, depleting the cell of an important precursor metabolite, resulting in cell growth arrest. The purpose of our study was to determine whether acetyl-CoA depletion occurs in the C584 strain incubated in M9 minimal media, and if so, whether sodium acetate, an extracellular source of acetyl-CoA, can replenish acetyl-CoA levels and allow for the growth of E. coli C584 in M9 minimal media. Our results suggest that intracellular acetyl-CoA levels decrease in E. coli C584 grown in M9 minimal media and that sodium acetate supplementation restores these levels to sufficient levels, enabling E. coli C584 growth.

Escherichia coli (*E. coli*) C584 and DH5α are two closely related strains, differing only in the presence of a pMOB3 plasmid in C584 and its absence in DH5α (3). The plasmid pMOB3 contains a mobilization (MOB) cassette to allow for interspecies exchange of the plasmid by conjugation, and kanamycin and chloramphenicol resistance genes for selection purposes (10). The chloramphenicol resistance gene product, chloramphenicol acetyl transferase (CAT) functions by catalyzing the transfer of the acetyl moiety from acetyl coenzyme A (acetyl-CoA) to a chloramphenicol molecule so that the antibiotic can no longer bind to ribosomes and inhibit protein synthesis (6, 11). In gram-negative bacteria such as *E. coli*, the *cat* gene is constitutively active and thus, even in media lacking chloramphenicol, it is still expressed (11). The CAT substrate, acetyl-CoA, is one of the precursor metabolites required for building block biosynthesis, particularly for the synthesis of fatty acids, sterols, and certain amino acids (7).

Kestell *et al*. (3) previously reported that, unlike the DH5α strain, C584 is unable to grow on M9 minimal media supplemented with glycerol and thiamine and that DH5α loses its ability to grow on this media when it is transformed with the pMOB3 plasmid, concluding that the plasmid is responsible for growth inhibition under limited nutrient conditions. Lau *et al*. (4) went on to show that when DH5α was transformed with a pMOB3 plasmid from which the *cat* gene had been deleted, the strain maintained its ability to grow on M9 minimal media. These results suggested that the *cat* gene is responsible for growth inhibition of pMOB3-transformed DH5α and presumably, of the C584 strain. Other groups have

also found that antibiotic resistance genes may inhibit growth (5, 8, 12). Potrykus and Wegrzyn (8) went on to show that CAT expressed in *E. coli* CM2555 inhibited growth by depleting intracellular acetyl-CoA levels.

Based on these findings, we hypothesize that the failure of *E. coli* C584 to grow on M9 minimal media is caused by CAT-mediated depletion of intracellular acetyl-CoA and that supplementation of this media with sodium acetate, a source of acetyl-CoA (2), will restore intracellular acetyl-CoA levels, allowing for growth of C584 on M9 minimal media.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The two *E. coli* strains, DH5α and C584, used throughout this experiment were supplied by Dr. William Ramey. The C584 strain is a DH5α strain transformed with a pMOB3 plasmid (3). Cultures were plated on Luria broth (LB) media and M9 minimal media ± 50 µg/ml chloramphenicol. All M9 cultures were supplemented with 0.2% glycerol and 0.5 µg/ml thiamine. Additional cultures were plated on the same media supplemented with 0.15% sodium acetate. All plated cultures were incubated aerobically in the dark at 37°C. The incubation times were 24 hrs for all plates and an additional 24 hrs if no growth were observed. All overnight cultures were grown in liquid LB media and incubated at 37°C with mild aeration.

Determination of bacterial growth rate. The growth rates for DH5α and C584 in M9 minimal media were determined by three separate time-course assays of turbidity measurements in the absence and presence of sodium acetate. The turbidity at 660 nm (OD_{660}) of overnight cultures grown in LB media was measured. The volume of culture required for an initial OD_{660} reading of 0.3 in 120 mls was calculated based on the assumption that 1 OD_{660} is equivalent to 1 x 10^7 cells/ml. The calculated amount of culture was centrifuged at 5000 x g for 10 mins to remove the LB media. Then the pellet was resuspended with 1 ml of M9 media and transferred to flasks containing 120 mls of M9 minimal media. The cultures were grown at 37°C with mild aeration. The initial OD_{660} for each culture was measured and subsequent readings were taken every 10 mins for a total of 12 time points. After the 6th time point, 0.15% sodium acetate was added to the media. Five mls of each culture was removed at each time point for the assay of the intracellular concentration of acetyl-CoA.

Preparation of cell-free extracts. Cell-free extracts were prepared by a modified version of the perchloric acid extraction (9) as performed by Potrykus and Wegrzyn (8). Harvested cells (5 ml) were centrifuged at 5000 x g for 10 mins, the supernatant discarded, and the pellet resuspended in 1 ml washing buffer composed of 10 mM biphasic sodium phosphate (pH 7.5), 10 mM $MgCl_2$, and 1 mM EDTA. Then the cell suspension was treated with ice-cold 3M $HClO_4$ (200 µl per ml of cell suspension). The mixture was incubated on ice for 30 mins and re-centrifuged at 5000 x g for 10 mins. The supernatant was removed and neutralized with saturated $KHCO_3$.

Determination of intracellular concentration of acetyl-CoA. To 0.45 ml of each sample, 150 mM Tris-HCl (pH 7.8), 10 mM malic acid, 3 mM $MgCl_2$, 7.55 mM NAD^+, 20 units/ml malate dehydrogenase, and distilled H_2O were added for total volume of 1 ml. The samples were incubated at 30°C for 30 mins and the initial optical density at 340 nm was measured. Citrate synthase (10 units/ml) was added to each sample and incubated for another 45 mins at 30°C. The final A_{340} was measured and the concentration of acetyl-CoA was calculated based on the equation (1),

$$0.322 \times \Delta E \times \frac{V}{v} = \mu\text{moles acetyl} - \text{CoA in the whole sample}$$

where ΔE = the difference in absorbance readings, V = the total volume of the sample, and v = the volume assayed.

TABLE 1. Growth properties of *E. coli* C584 and DH5α in LB media and M9 minimal media in the presence or absence of chloramphenicol (CAM) were tested in three replicates.

Strain	Media[a]		Replicates	
		1	2	3
C584	LB	+	+	+
	LB + CAM	+	+	+
	M9	-	-	-
	M9 + CAM	-*	-*	-
DH5α	LB	+	+	+
	LB + CAM	-	-	-
	M9	+	+	+
	M9 + CAM	-	-	-

+ = Growth observed

- = No growth observed

[a] M9 minimal media was supplemented with 0.2% glycerol and 0.5μg/ml of thiamine.

* Growth of contaminants observed on these plates.

RESULTS

To test our hypothesis that *E. coli* C584 growth inhibition on M9 media is caused by an intracellular depletion in acetyl-CoA by the activity of the CAT protein encoded by the *cat* gene on the pMOB3 plasmid, we first sought to confirm the previous finding by Lau *et al*. (4). *E. coli* C584 and DH5α strains were plated onto various media in three replicates and tested for their ability to grow (Table 1).

The plating results indicated the ability of C584 to grow on LB media with and without chloramphenicol. The ability of *E. coli* C584 to grow on media containing chloramphenicol also confirmed the presence of the pMOB3 plasmid containing the *cat* gene. When *E. coli* C584 was plated on M9 media with and without chloramphenicol, no growth was observed. Growth was actually observed in two replicates of M9 media containing chloramphenicol for the C584 strain. However, microscopic analysis and visual observation of these colonies indicated that they were contaminants. These contaminants were filamentous rather than rod shaped like *E. coli*. Interestingly, contaminants of this nature were also observed by Lau *et al*. in their plating experiments (4). Additional M9 + CAM plates were plated to confirm this observation and no growth was observed (data not shown). DH5α plating indicated its ability to grow on both LB and minimal media without chloramphenicol. No growth of DH5α was observed in media containing chloramphenicol.

To determine whether a lack of acetyl-CoA was the cause of growth inhibition, C584 and DH5α were plated on various media with sodium acetate (Table 2), which serves as a source of acetyl-CoA (2).

TABLE 2. Growth properties of *E. coli* C584 and DH5α in LB media and M9 minimal media with 0.15% sodium acetate (NaAc) in the presence or absence of chloramphenicol (CAM) were tested in three replicates.

Strain	Media[a]		Replicates	
		1	2	3
C584	LB + NaAc	+	+	+
	LB + CAM + NaAc	+	+	+
	M9 + NaAc	+	+	+
	M9 + CAM + NaAc	+	+	-*
DH5α	LB + NaAc	+	+	-*
	LB + CAM + NaAc	-	-	-
	M9 + NaAc	+	+	+
	M9 + CAM + NaAc	-	-	-

+ = Growth observed

- = No growth observed

[a] M9 minimal media was supplemented with 0.2% glycerol and 0.5 µg/ml of thiamine.

* No growth was observed, which was not consistent with other replicates.

Addition of sodium acetate to M9 minimal media, with and without chloramphenicol, was able to remove the growth inhibition of C584 on M9 minimal media. This result indicated that the lack of acetyl-CoA was a major contributing factor in the growth inhibition of C584 in M9 minimal media. On the other hand, the growth pattern of *E. coli* DH5α was similar on both types of media with and without sodium acetate but no growth was observed when chloramphenicol was present in the media. A few replicates were observed to be inconsistent with others, in that no growth was observed. These inconsistencies were most likely the result of human errors.

The plating results indicated that the addition of sodium acetate removes growth inhibition of the C584 strain on M9 minimal media. We were interested in further confirming our hypothesis by monitoring the intracellular acetyl-CoA concentration of C584 and DH5α with respect to time. Three time trial assays were performed and each time trial measured the optical density of the cells at 660nm and intracellular acetyl-CoA concentration every ten minutes for one hour and twenty minutes (Fig. 1 a & b).

The intracellular acetyl-CoA concentration in *E. coli* DH5α might have dropped following the addition of external acetate but remained fairly steady throughout the time trial. The turbidity of DH5α increased throughout the time trial and did not appear to be limited by the availability of a source of acetyl-CoA. On the other hand, the intracellular acetyl-CoA concentrations in C584 showed a decrease after transfer from LB media to M9 minimal media. Then following addition of sodium acetate at 50 mins, the intracellular acetyl-CoA concentration increased. The turbidity showed a steady reading from 0 to 70 mins before gradually increasing again. The set point of acetyl-CoA levels in DH5α appears to be higher than C584, as indicative of the difference seen at time point zero.

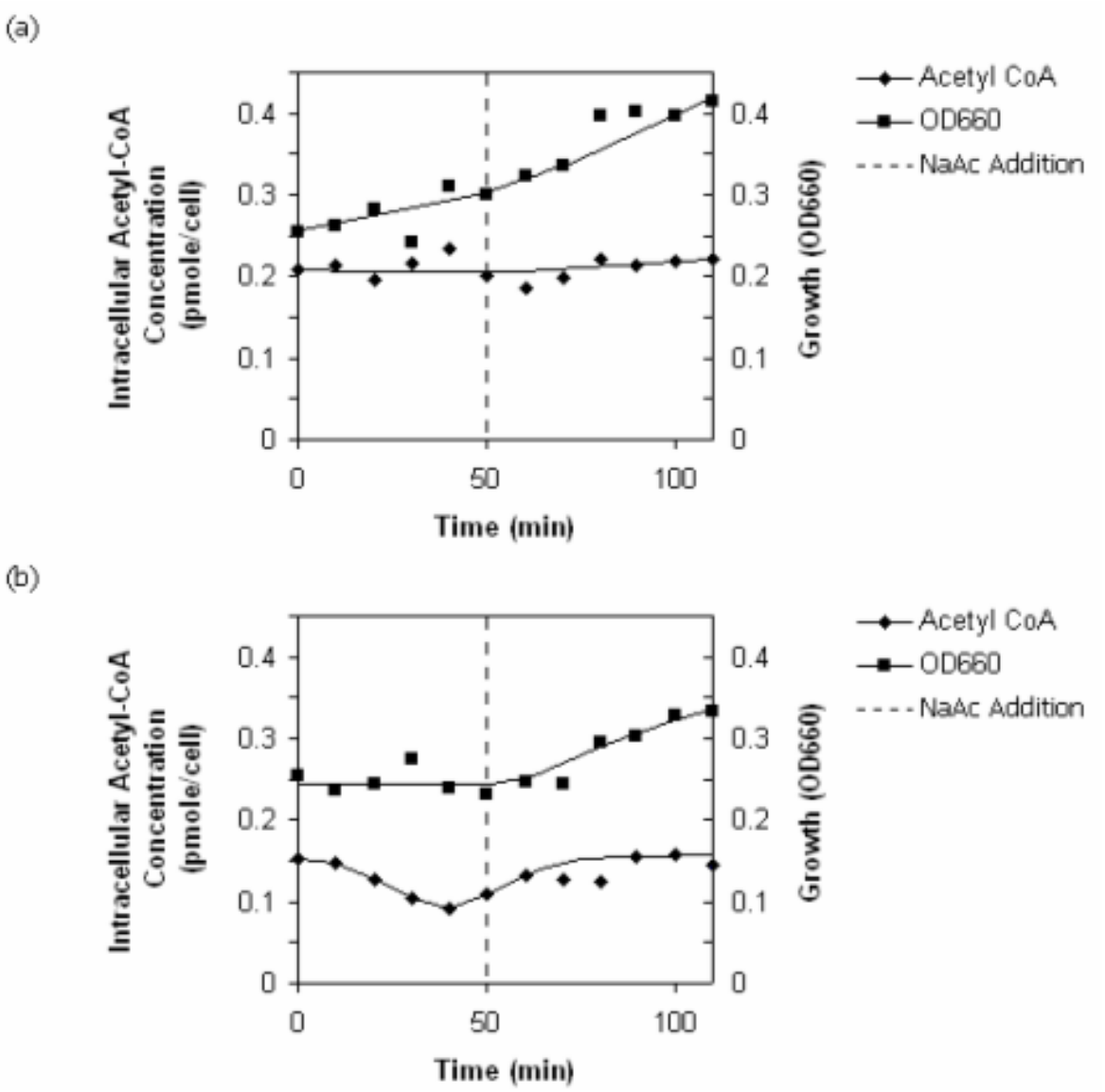

FIG. 1 Intracellular amounts of acetyl-CoA as affected by time in M9 minimal media for *E. coli* (a) DH5α (b) C584. Strains were grown in LB media and transferred to M9 minimal media at time zero to an adjusted OD_{660} of 0.3. Samples were drawn every 10 min after transfer of cells in M9 minimal media and were then assayed for OD_{660} and acetyl-CoA. At 50 min, 0.18g of sodium acetate was added to the 120 mls of media (0.15% final concentration).

DISCUSSION

The plate growth patterns confirmed the findings of Lau *et al*. (4) as *E. coli* C584 failed to grow on M9 media with or without the presence of chloramphenicol, while *E. coli* DH5α did grow on M9 medium without antibiotic (Table 1). We also found that in most cases, supplementation of M9 media with sodium acetate reversed the growth inhibition of C584, suggesting that acetyl-CoA was lacking in growth-inhibited C584 and that sodium acetate supplementation was sufficient to restore acetyl-CoA to adequate levels to allow for growth. The two cases in which plate growth results deviated from those of the other replicates are likely due to experimental error.

The plating results were consistent with our hypothesis that CAT depletes intracellular acetyl-CoA, which cannot be replenished with glycerol as the sole carbon source. An exogenous source of acetyl-CoA, in the form of sodium acetate, was sufficient to offset the depletion by CAT allowing for C584 growth on M9 minimal media (Table 2). Since the growth patterns were nearly identical in the sodium acetate-supplemented M9 minimal media with or without chloramphenicol, it appeared that the action of CAT was independent of the presence of chloramphenicol. However, this was inconsistent with the known mechanism of action of CAT which involves the transfer of the acetyl moiety from acetyl-CoA to a chloramphenicol molecule (6). Our results suggest that CAT depletes acetyl-CoA even when chloramphenicol is not present to accept the acetyl moiety. This observation suggests that another unknown molecule may be accepting the acetyl moiety in place of chloramphenicol. The possibility of an alternate substrate for CAT should be investigated further.

The results of our assay for intracellular acetyl-CoA (Fig.1) were consistent with the results obtained from our plating (Table 2). Although three time assays were performed, only the data for the most representative trial is shown. DH5α was used as the control for our assay and because this strain demonstrated the ability to grow on M9 minimal media, we believed that the amount of acetyl-CoA in DH5α would remain at levels sufficient for growth. The starting OD_{660} for both cultures was approximately 0.26, which was lower than the calculated OD_{660} of 0.3. This difference was likely due to the fact that the calculations for an initial OD_{660} of 0.3 was based on readings taken when the cells were still growing in LB media, which was transparent and yellow in color. Subsequent readings were taken when the cells were growing in M9 minimal media, which was colorless. The color of the LB media likely contributed to the underestimation of the OD_{660} in colorless M9 minimal media.

The initial level of acetyl-CoA in DH5α was higher than the level in C584. These levels were measured immediately after the transfer of cells from rich media to minimal media, indicating that the intracellular level of acetyl-CoA in C584 was naturally lower. Since the only difference between the two strains was the presence of the pMOB3 plasmid in C584, the reduced levels of acetyl-CoA in C584 were likely due to the action of CAT, encoded by *cat* gene on pMOB3. We assumed that the amount of acetyl-CoA in strain DH5α would remain steady because these cells would be able to replenish its pools of acetyl-CoA. This assumption was not supported by our results (Fig.1a). The level of acetyl-CoA in DH5α appeared to decrease after transfer to M9 minimal media. This can be attributed to the fact that the cells were removed from a rich environment and placed into an environment where nutrients were lacking, which resulted in a shift between uptake of nutrients and production of acetyl-CoA. When DH5α was introduced into the minimal medium, the cells were likely using acetyl-CoA at the same rate as in rich media. However, because of the lack of nutrients available in M9 minimal media, these cells could not replenish acetyl-CoA as before, resulting in the decrease we observed. The decreasing levels of acetyl-CoA would eventually reach a new state of equilibrium in DH5α, which was not low enough to inhibit its growth as the optical density of DH5α increased steadily after transfer into minimal media. Addition of exogenous sodium acetate appeared to have slightly increased the growth of these cells and restored the level of acetyl-CoA to similar levels as in rich media.

After transfer of C584 from rich media to minimal media, the same reduction of acetyl-CoA levels in C584 was expected; however, the decrease we saw in C584 was more drastic and thus cannot be attributed to the changing of media alone. Growth of C584 in M9 minimal media resulted in a decrease in the amount of intracellular acetyl-CoA prior to the addition of sodium acetate (Fig. 1b). The optical density readings at 660nm appeared to be approximately constant, fluctuating around a reading of about 0.25, suggesting that no growth occurred before the addition of sodium acetate. Because the decrease in intracellular acetyl-CoA coincided with no growth in strain C584, we suspected there was a causal relation between the two. Our suspicions appeared to be confirmed after the addition of sodium acetate to the media. After the addition of exogenous sodium acetate at 50mins, the optical density readings steadily increased indicating that a degree of growth had been restored. As well, the amount of intracellular acetyl-CoA increased to a concentration similar to that observed in rich media. We hypothesize that CAT depleted the acetyl-CoA in C584 faster than the cell could replenish its stocks. Because acetyl-CoA is the central compound in the citric acid cycle and in the formation of various biosynthetic precursors, its depletion would result in a decrease of various essential molecules, such as fatty acids and sterols (7), causing growth inhibition. By adding an exogenous source of acetyl-CoA, the production of these essential molecules was restored, which in turn restored C584 growth (Fig. 1b).

From the experiments conducted by Kestell *et al.* (3) and Lau *et al.* (4), we know that elements on the pMOB3 plasmid, principally the *cat* gene, are involved in the growth inhibition of *E. coli* strain C584 on M9 minimal media. However, Lau *et al.* (4) only performed a loss of function experiment for the *cat* gene and were not able to conclusively demonstrate through a gain of function experiment that the *cat* gene was the only element of pMOB3 that was involved.

Through our experiment, we were able to show that the *cat* gene is involved in the depletion of the intracellular concentration of acetyl-CoA in strain C584. We believe that the CAT enzyme either transfers the acetyl moiety of acetyl-CoA to another molecule in the cell or alters the molecule in such a way that it can no longer be utilized by the cell. The depletion of intracellular acetyl-CoA in C584 is significant enough to prevent growth in M9 minimal media, thus, an extracellular source of acetyl-CoA is required to support growth. We did not determine if C584 growth is inhibited in other types of minimal media or if this phenomenon of growth inhibition is specific for M9 minimal media only. We suspect this growth inhibition will also be seen in other minimal media; however, further experimentation will have to be performed to determine whether specific elements of the M9 minimal media are involved in growth inhibition.

FUTURE EXPERIMENTS

Future experiments can confirm the *cat* gene on pMOB3 as the gene responsible for the chloramphenicol resistance. The previous paper (4) had not conclusively demonstrated that the *cat* gene is the sole gene responsible for this growth inhibition. We propose that experiments focus on the excision and isolation of the *cat* gene from pMOB3 followed by its insertion into an irrelevant transformation vector. This new construct should be used to transform DH5α and successful transformants should be tested for their ability to grow on media containing chloramphenicol. By doing this experiment, other components of the pMOB3 plasmid can be conclusively ruled out as

conferring resistance to chloramphenicol.

In our experiments, we saw filamentous contaminants in C584 plated on M9 minimal media with chloramphenicol. Lau *et al*. also saw the same contaminants in their experiment, but in all their plates. This observed similarity in contaminant morphology leads us to suspect that it may be too coincidental to be a simple contamination. Possible explanations for these contaminant-like growths include altered state of growth of C584 under stressed conditions. It would be of interest to re-isolate this contaminant and determine its true identity through staining, biochemical tests, and even rRNA sequencing. If it is truly a contaminant, its origin should be investigated.

Lastly, the growth inhibition of *E. coli* C584 has only been studied in M9 minimal media. Future experiments can grow C584 on other types of minimal media to see if this phenomenon of growth inhibition is also observed or if it is specific for M9 minimal media. If growth inhibition of the C584 strain is only seen in M9 minimal media, then it may be possible that M9 minimal media contains elements which contribute to the growth inhibition.

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