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The Effect of *Plectranthus barbatus* derived Forskolin on Cyclic 3' 5' Adenosine Monophosphate Levels measured as β-galactosidase activity and on Glucose Transport in *Escherichia coli* B23

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The labdene diterpene forskolin acts on the enzyme adenylyl cyclase to increase cyclic-3' 5' adenosine monophosphate (cAMP) levels and is thought to have an effect on glucose transport in eukaryotic cells. Previous studies have shown this compound to act on mammalian cells, however studies concerning the effect of forskolin on *E. coli* is limited. Characterizing potential effects could provide a method for cAMP modulation in *E. coli* without exogenous cAMP addition. This study aimed to determine the effect of forskolin on *E. coli* B23 cells by examining potential changes in cAMP levels upon addition of this compound, and any potential effect on glucose transport. Results show that forskolin does not inhibit glucose transport as growth rate was not repressed in forskolin-supplemented cultures. Also, forskolin does not have a significant effect on β-galactosidase levels in *E. coli* B23, regardless of the carbon source utilized, and thus may not provide a reliable method for cAMP modulation in a research setting.

Forskolin, a labdene diterpene from the plant *Plectranthus barbatus*, is used clinically as a vasodilator, a potential weight loss aid, and to promote nerve repair in humans (9). By acting on the enzyme adenylyl cyclase to increase cyclic-3' 5' Adenosine Monophosphate (cAMP) levels, forskolin functions to re-sensitize cells to hormones and extracellular signals (3). Also, forskolin has been shown to have an effect on glucose transport within eukaryotic cells (3,5,7,10). Previous studies have shown this compound to act in tissue culture by inhibiting the GLUT1 transporter and on the Gram-negative bacteria *Xanthomonas campestris* by increasing cAMP levels through adenylyl cyclase modulation (10,15). The effect of forskolin on *Escherichia coli* (*E. coli*) is not well known. Characterizing these effects could provide a method for cAMP modulation in *E. coli* without exogenous addition.

This study aimed to determine the effect of forskolin on *E. coli* B23 cells by examining potential changes in cAMP levels upon addition of this compound, and any potential effect on glucose transport. This was determined by quantifying β-galactosidase (β-gal) levels under different experimental conditions, including growth in glucose, glycerol, and pyruvate with or without the addition of forskolin. β-gal levels are indicative of cAMP production as cAMP acts as a transcriptional regulator of the β-gal encoding Lac operon (8). β-gal activity was quantified by measuring the absorbance of nitrophenol, a coloured substrate generated by the enzymatic cleavage (mediated by β-gal) of ONPG by β-gal. To determine the effect of forskolin on glucose transport within cells, a growth curve was performed on *E. coli* B23 cultures provided with glucose as the sole carbon source. Results indicate that forskolin does not inhibit glucose transport as growth rate was not repressed in forskolin treated cultures. Also, forskolin does not have a significant effect on β-gal levels in *E. coli* B23, regardless of the carbon source utilized, and thus may not provide a reliable method for cAMP modulation in a research setting.

MATERIALS AND METHODS

Materials. *Escherichia coli* B23 (MICB 421 teaching laboratory frozen stock, University of British Columbia). Forskolin (LC Laboratories, Cat # F-9929). Isopropyl-β-D-thiogalactopyranoside (IPTG; supplied stock solution, UBC Teaching Laboratories). Glycerol (Life Technologies, Cat # 15514-011)). L-Glucose (supplied stock solution, UBC Teaching Laboratories). Sodium Pyruvate (Sigma Chemical Company, St. Louis, MO, Cat # S9378). o-Nitrophenyl-β-D-galactopyranoside (ONPG; supplied stock solution, UBC Teaching Laboratories). Toluene (Fisher Scientific). TM Buffer ((Tris (pH 8.0, 10mM), magnesium chloride (0.1 mM)), supplied stock solution, UBC Teaching Laboratories). Sodium carbonate (supplied stock solution, Fisher Scientific). Ethanol (98%, UBC Teaching Laboratories).

Bacterial Strains and culturing. *E. coli* B23 were grown over 27 hours in M9 minimal media (10) supplemented with 0.4% w/v glucose at 37°C under mild aeration (150 rpm) in a shaking water bath. A 500µl aliquot of the overnight culture was added to each of three flasks containing 50 ml of M9 minimal media supplemented with either glucose (0.4% w/v), glycerol (0.4% w/v) or pyruvate (0.4% w/v). Inoculated flasks were grown over 27 hours at 37°C under mild aeration (150 rpm) in a shaking water bath. Six flasks containing 50 ml of M9 minimal media supplemented with either glucose (0.4% w/v), glycerol (0.4% w/v), or pyruvate (0.4% w/v) were inoculated with a sufficient aliquot of an overnight (18 hours) culture grown in the same sugar to obtain a starting turbidity of 0.25 OD_{460} units. Culture flasks were placed in a 37°C shaking water bath with mild aeration (150 rpm) until sampling began.

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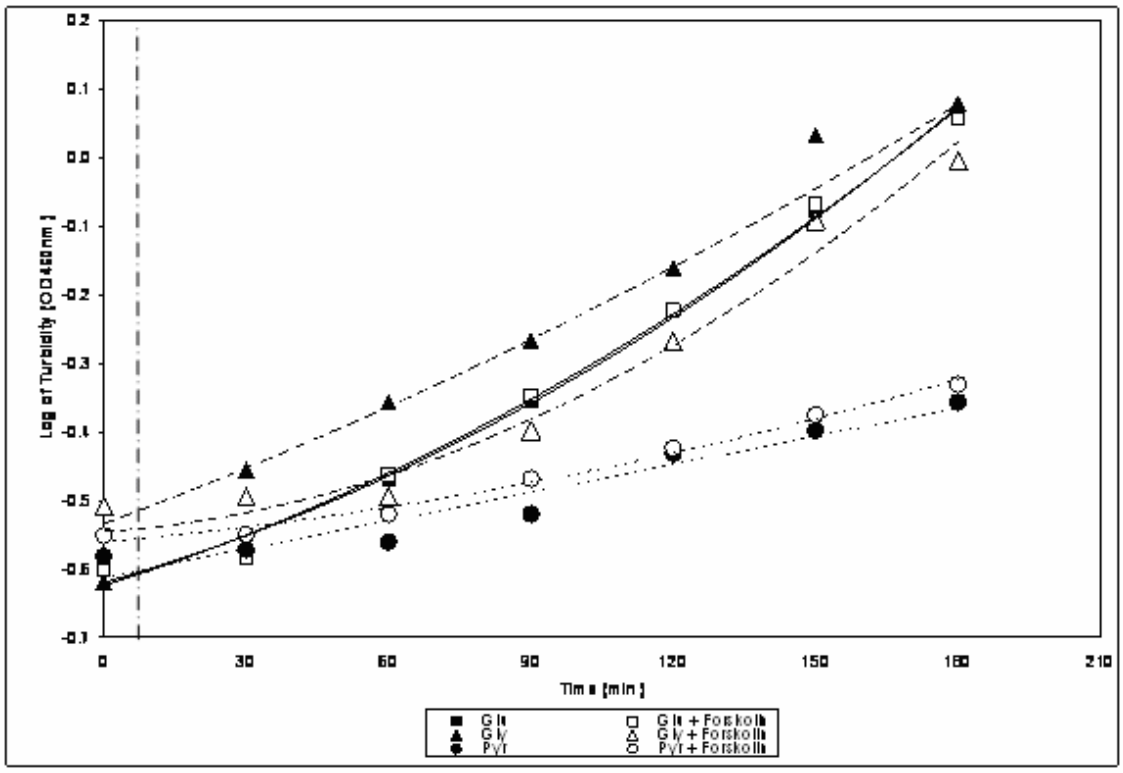

FIG. 1. Effect of forskolin on growth of *E. coli* B23 cells. Grown in M9 minimal media with either glucose, glycerol, or pyruvate at 37ºC in a shaking water bath. All cultures induced with IPTG at time 0 and experimental cultures have addition of 150 µM forskolin at 10 minutes (dashed vertical line represents addition of forskolin).

Sampling and growth assay. A 3ml aliquot was withdrawn from each culture flask and timing was initiated. All cultures were induced with IPTG (final concentration of 300 µg/ml) immediately after initial sampling. Forskolin (at a final concentration of 150 uM) was added to 3 flasks, each representing a different sugar, at 10 minutes after initial sampling. Three ml samples were removed every 30 minutes after initial sampling, up to 180 minutes. Cultures were maintained at 37°C and aerated at 150 rpm throughout sampling. Turbidity (at 460nm) to assess growth of each 3 ml aliquot was immediately read on a Spectronic 20 spectrophotometer after removal from culture flasks. Sample growth was then stopped by the addition of 100 µl toluene and subsequent vortexing (60 seconds). The lower phase of toluenized samples was removed and centrifuged for 2 minutes at 13 000 Xg to pellet cells. Supernatant was removed and cell pellets were placed on ice for the course of the experiment then frozen at -80°C until they were assayed for activity.

Assay for β-galactosidase. Cell pellets were thawed in a 37°C water bath for 10 minutes and subsequently re-suspended in 1.1ml of TM buffer. Samples were again warmed in a 37°C water bath for 5 minutes. Two hundred ul of 5 uM ONPG was added, followed by 2 ml of 0.6 M sodium carbonate after 1 minute of color development. Absorbance at 420 nm was measured using a Spectronic 20 spectrophotometer for each sample. Results were converted to enzyme activity per mL of supplied pellet using the following equation: (A/t) X (10^6/15,000) X (Nv/Ev) (12).

RESULTS

Effect of forskolin on growth. The growth of *E. coli* B23 in glucose M9 minimal media was not affected by the addition of forskolin (Fig. 1). The growth rate was nearly identical at all time points when comparing the control and forskolin-supplemented cultures (Fig. 1). *E. coli* B23 cultures in glycerol appeared to show an initial lag in the presence of forskolin then grew at the same rate as the control. However, a difference in initial optical density at 460nm between the control and forskolin treated culture may indicate the potential for a large degree of error. All subsequent time points may be affected by that error. Cultures grown in pyruvate alone showed no significant difference in growth rate when compared to forskolin treated cells (Fig. 1). In general, no clear difference in growth rate was seen with the addition of forskolin to *E. coli* B23 cultures, regardless of the carbon source (Fig. 1).

As expected, cultures grown in both glucose and glycerol exhibited similar growth rates, while growth rate in pyruvate is 2.5 times lower (Fig. 1). All cultures displayed an average initial optical density of 0.25 at 460nm, and did not reach stationary phase during the 180 minute time course.

Effect of forskolin on glucose transport. The growth rates exhibited by glucose and glucose-supplemented forskolin *E. coli* B23 cultures were

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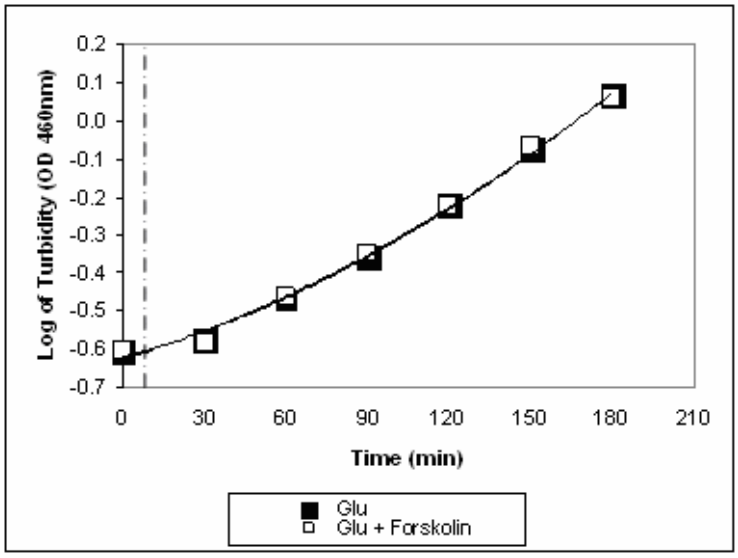

FIG. 2. Effect of forskolin on glucose transport assessed by the effect on growth of *E. coli* B23 cells. Incubated in M9 minimal media with glucose at 37ºC in a shaking water bath. Both cultures induced with IPTG at time 0 and the experimental culture had addition of 150 µM forskolin at 10 minutes (dashed line indicates addition of forskolin).

identical (Fig. 2.). This indicated that forskolin did not inhibit glucose transport. This was unexpected.

Effect of forskolin on β-galactosidase levels. Cultures grown in glucose with forskolin showed no significant difference in β-gal levels compared to cultures grown in glucose alone. An increase in enzyme activity was observed between 90 and 150 minutes (Fig. 3A), however this trend might not be reliable due to the level of possible error suggested by the 0 minute time point.

Cultures grown in glycerol alone initially exhibited β-gal levels 2 times greater then glycerol cultures treated with forskolin. However, later time points showed cultures grown in glycerol with the addition of forskolin exhibit increased β -gal levels relative to the control (Fig. 3B). A more pronounced increase in production over the 180 minute time course was also seen in forskolin-treated glycerol cultures when compared to cultures grown in glycerol alone (Fig. 3B). Though a marked increase in β-gal levels was observed in the forskolin-treated culture, a substantial difference in initial enzyme activity at the zero minute time point suggests a high probability of large potential error in the dataset.

The pyruvate grown culture did not show any consistent observable differences when treated with forskolin, as compared to the control culture (Fig. 3C).

DISCUSSION

The growth assay showed that *E. coli* B23 grown in pyruvate exhibited a 2.5 times slower growth rate than *E. coli* B23 grown in glucose and glycerol (Fig. 1). The results of the growth assay showed expected trends (4,16). Forskolin did not appear to affect the growth rate of *E. coli* B23 grown in glucose, glycerol or pyruvate. This implies that forskolin did not inhibit the transport of any carbon sources tested. This was unexpected, as it was previously shown that forskolin inhibits glucose transport in mammalian cells (14). However, the eukaryotic and prokaryotic glucose transporters vary in both structure and function. This could explain why glucose transport was not inhibited by forskolin in *E. coli* B23. In *E. coli* B23 the glucose transporter utilizes a phosphotransferase system (PTS) whereby a phosphate derived from phosphoenolpyruvate (PEP) is sequentially transferred through the components of the PTS (11). This results in the formation of glucose-6-phosphate (11). In contrast, the mammalian glucose transporter (GLUT1) functions via passive transport of glucose (10). The mammalian GLUT1 is homologous to the E. coli galactose-H+ transport protein, GalP (10). The GalP transporter is required for the transport of galactose. However, in this study the effects of forskolin on galactose transport were not investigated.

In previous studies, *E. coli* cultures grown in glycerol and glucose were shown to have similar growth rates, while cultures grown in pyruvate exhibited a significantly lower growth rate (1). In this study, initial growth assay experiments showed unexpected results (4,16). *E. coli* B23 cells grown in the presence of pyruvate showed the highest growth rate when compared to cells grown in glucose and

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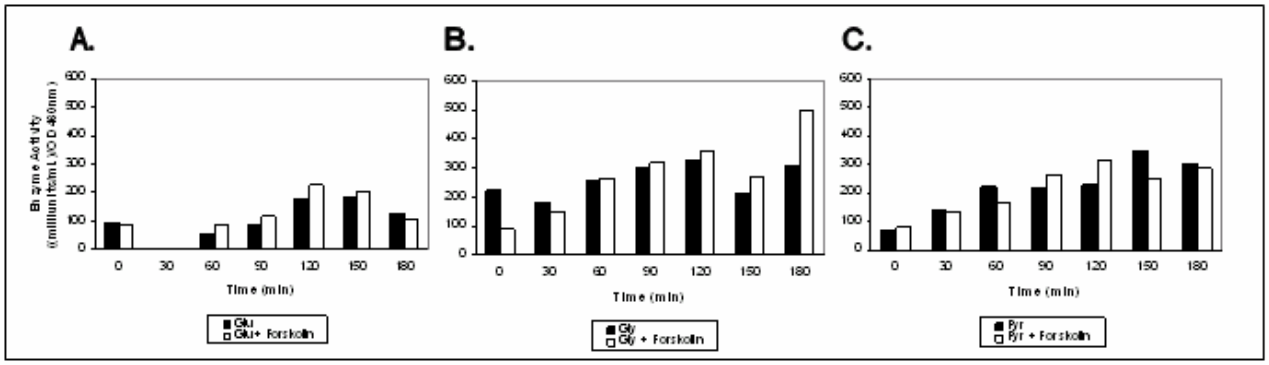

Fig.3. Effect of forskolin on β-galactosidase levels in *E. coli* B23 cells grown in M9 minimal media with (A) glucose, (B) glycerol, and (C) pyruvate at 37ºC in a shaking water bath. All cultures were induced with IPTG at time 0 and experimental cultures were supplemented with 150 μM forskolin at 10 minutes.

glycerol (data not shown). Because it has been previously established that pyruvate is a poor carbon source and should have a lower growth rate (1), the initial data obtained was omitted.

β-gal expression in control cultures grown in glycerol, glucose and pyruvate show previously reported trends, with glycerol yielding the highest β-gal levels, followed by pyruvate and glucose (4). In this study, cultures grown in glycerol exhibited the highest β-gal levels at an average of 303 milliunits mL^{-1} OD_{460nm}^{-1} when compared to cultures grown in glucose (average of 119 milliunits mL^{-1} OD_{460nm}^{-1}) or pyruvate (average of 255 milliunits mL^{-1} OD_{460nm}^{-1}). This difference is due to the initiation of catabolite repression by glucose, subsequently resulting in the inhibition of adenylyl cyclase and cAMP levels. Depletion of the phosphate pool by pyruvate also leads to a decreased level of cAMP and β-gal. Pyruvate conversion to phosphoenol pyruvate in the process of gluconeogenesis depletes the phosphate pool, leaving less available to generate cAMP (17). All three carbon sources lead to a maximum level of β-gal at approximately 120 minutes, with a slight decrease thereafter. This decrease was caused by maintenance of constant β-gal expression while growth continued exponentially.

In general, no conclusive evidence of the effect of forskolin on β-gal levels in *E. coli* B23 cultures was observed. In the culture grown in glucose supplemented with forskolin, a slight increase of β-gal levels is seen between the 90 and 150 minute time points (Fig. 3A). This increase could be due to the effects of forskolin on cAMP or it could be a result of the inherent error present within the dataset. To better characterize these effects experimental replicates must be performed. A significant amount of error is affiliated with the glycerol cultures (with or without forskolin), therefore no trends can be ascertained. However, disregarding the significant amount of error it appears that the forskolin-supplemented glycerol culture has an increased rate of β-gal expression when compared to the control. This could be evidence supporting the hypothesized effect of forskolin on adenylyl cyclase causing an increase in cAMP levels, resulting in increased β-gal levels. In pyruvate grown cultures (with or without forskolin) no significant effect of forskolin is seen. Pyruvate causes depletion of the phosphate pool, as previously mentioned, and this effect could override any potential effect of forskolin on adenylyl cyclase.

Studies on forskolin are significant as they may provide a method for cAMP modulation in *E. coli* without exogenous addition. Control of endogenous cAMP production allows the regulatory system to be effected through adenylyl cyclase. This is important as the activation of adenylyl cyclase through phosphorylation may be contributing to the activation of other biological pathways which work in conjunction with cAMP (2). With exogenous addition, these pathways may not be activated and the observed effect of cAMP on cells would not be representative of a natural state. This is exemplified by a study on *E. coli* chemotaxis which suggests that exogenous addition of cAMP is not sufficient to restore chemotactic response in adenylyl cyclase deficient mutants (2). Thus, it is the adenylyl cyclase itself that is key in many biological processes including control of the lac operon and chemotaxis. Because forskolin may provide a direct way to modulate adenylyl cyclase rather then simply cAMP levels, this compound could allow for the observation and manipulation of bacterial processes through a more natural mechanism.

Overall, forskolin was not shown to inhibit glucose transport, as inferred through a constant growth rate between control and forskolin treated cultures. Furthermore, any effect of forskolin on cAMP levels can not be ascertained from this study due to high probability of error and lack of biological replicates.

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FUTURE EXPERIMENTS

Due to time constraints this study was only performed once and should be repeated in order to verify results. This study did not determine whether forskolin has an effect on cAMP levels. This could be further investigated by using a range of higher forskolin concentrations in both the growth assay and enzyme assay. Forskolin may also be added prior to the induction with IPTG to maximize the time for the forskolin to act on cAMP levels. In order to show whether the trends observed are reproducible, biological replicates would be required. Specifically, it would be of interest to determine if forskolin inhibits galactose transport as previously reported, this would also help determine the effective in vivo concentrations of forskolin (10). Levels of cAMP and possible ctabolite repression by galactose could also be studied as forskolin has been shown to only inhibit the GalP transporter (required for galactose transport) out of the six sugar-H^+ symport systems in *E. coli* (10). In the future, standard concentrations of β-gal can be used to measure the degree of variability contained within the enzyme assays.

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