dye (*arc*) mutants: insights into an unexplained phenotype and its suppression by the synthesis of poly (3-hydroxybutyrate) in *Escherichia coli* recombinants

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Abstract

arcA codes for a central regulator in *Escherichia coli* that responds to redox conditions of growth. Mutations in this gene, originally named *dye*, confer sensitivity to toluidine blue and other redox dyes. However, the molecular basis for the dye-sensitive phenotype has not been elucidated. In this work, we show that toluidine blue redirects electrons to O_2 and causes an increase in the generation of reactive O_2 species (ROS). We also demonstrate that synthesis of poly (3-hydroxybutyrate) suppresses the Dye phenotype in *E. coli* recombinants, as the capacity to synthesize the polymer reduces sensitivity to toluidine blue, O_2 consumption and ROS production levels.

Introduction

Escherichia coli and other Enterobacteria can adapt their metabolism to O_2 availability in the environment. Aerobic and anaerobic respiration and fermentation are different metabolic pathways that enable bacteria to optimize energy generation according to O_2 levels in the growth medium (Patschkowski *et al.*, 2000). Sophisticated and interrelated regulatory networks switch on and off the expression of these pathways as needed, in order to optimize bacterial metabolism according to the availability of suitable electron acceptors. One of these regulatory systems, the ArcAB two-component signal transduction system, modulates the expression of many operons according to the redox conditions of growth at the transcriptional level. ArcB is a transmembrane sensor kinase, which under low O_2 concentration conditions undergoes autophosphorylation and then transphosphorylates the response regulator ArcA, one of the global regulators in *E. coli*. The main targets for repression by the phosphorylated regulator are the genes that code for the enzymes involved in aerobic respiration, such as those of the tricarboxylic acid cycle. On the other hand, in low O_2 conditions ArcA activates respiratory chain enzymes such as the cytochrome *d* oxidase, with high affinity for O_2, and fermentation enzymes such as pyruvate–formate lyase.

The *arcA* gene was first detected by the spontaneous appearance of small colonies in *E. coli* cultures plated on eosin methylene blue medium, usually used for the detection of *lac* mutants, and originally named *dye* (Roeder & Somerville, 1979). It was observed that mutations in this gene conferred sensitivity to dyes such as methylene blue and toluidine blue (TB), and later it was also related to male sterility in Hfr, F^+ and F′ strains (Buxton & Drury, 1983). However, the molecular basis for the dye-sensitive phenotype remained unknown, although it was hypothesized that in the *arc* mutants the electron transport chain is altered in a way that allows dyes to divert electrons in a futile flow (Lynch & Lin, 1996).

Many bacterial species synthesize the highly reduced polyhydroxyalkanoic acids (PHAs), which accumulate in the cytoplasm as hydrophobic granules (Madison & Huisman, 1999) and function as carbon and electron sinks that enhance survival and stress tolerance in bacteria (Okon & Itzigsohn, 1992; Ruiz *et al.*, 2001). These thermoplastic

polymers have drawn great interest since their discovery, due to their biodegradability and the potential to produce them from renewable carbon sources (Madison & Huisman, 1999). The genes responsible for poly(3-hydroxybutyrate) (PHB) biosynthesis (*pha* genes) in *Azotobacter* sp. strain FA8 have been cloned in our laboratory, and shown to promote poly(3-hydroxybutyrate) accumulation in *E. coli* recombinants from several carbon sources (Pettinari *et al.*, 2001; Nikel *et al.*, 2005).

Our hypothesis was that the heterologous expression of *pha* genes in *E. coli arc* mutants could funnel the excess reducing power generated towards intracellular bodies, thus suppressing the Dye phenotype.

The *E. coli arc* mutants are unregulated for aerobic respiration, and have an increased rate of O_2 consumption, resulting in the generation of larger quantities of O_2 radicals (Nystrom *et al.*, 1996). In this work, we demonstrate that TB redirects electrons to O_2, and causes an increase in the generation of reactive O_2 species (ROS). We also corroborate that synthesis of poly(3-hydroxybutyrate) suppresses the Dye phenotype, as the capacity to synthesize the polymer reduced sensitivity to TB, O_2 consumption and ROS production levels in *E. coli arc* mutants.

Materials and methods

Bacterial strains

The *Escherichia coli* strains used throughout this study are listed in Table 1. Strains that synthesize poly(3-hydroxybutyrate) carry plasmid pJP24. This plasmid was constructed by cloning an amplification fragment containing the *pha* structural genes from *Azotobacter* sp. FA8 under the control of the *lac* operator in expression vector pQE32 (Qiagen, Hilden, Germany), carrying ampicillin resistance (P.I. Nikel, A. de Almeida, E.C. Melillo, M.A. Galvagno & M.J. Pettinari, unpublished data).

Media and growth conditions

Cultures were routinely grown at 37 °C with reciprocal agitation (130 strokes per min) in Luria–Bertani (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, pH 7.2). When necessary, ampicillin was used at a concentration of 100 μg mL^{-1}. For poly(3-hydroxybutyrate) accumulation, *E. coli* strains carrying the *phaBAC* genes of *Azotobacter* sp. FA8 were cultured in LB medium supplemented with 1% xylose and 1 mM isopropyl β-D-thiogalactosidase (IPTG) to induce *pha* genes expression.

DNA manipulation and analysis

Plasmid DNA preparation, DNA ligation, bacterial transformation and agarose gel electrophoresis were performed following standard methods (Sambrook *et al.*, 1989) and instructions from the manufacturers. Restriction enzymes were purchased from Promega (Madison, WI). All plasmid constructions were propagated in *E. coli* DH5α.

Cloning of *arcA* for complementation studies

The *arcA* gene was amplified by PCR using the *E. coli* DH5α genome as a template and two oligonucleotides (5′-CCG AAA ATG AAA GCC AGT A-3′ and 5′-GAA AGT ACC CAC GAC CAA G-3′) located at positions 4128 and 5121 of section 400 of the complete genome of *E. coli* K12. The PCR reactions were performed in a final volume of 50 μL using 50 ng of template, 0.2 mM dNTPs, 0.75 μM of each primer, 2.5 mM $MgCl_2$, 1 × Taq amplification buffer and 0.5 U of Taq polymerase (Artec, Tecnolab, Buenos Aires, Argentina). Amplification was performed using standard protocols (annealing temperature 55 °C, elongation temperature 72 °C, 35 cycles). A 1012 bp DNA fragment corresponding to the entire *arcA* and the region 170 bp upstream of the initiation codon was obtained. The amplification fragment was ligated into vector pGEM-T Easy (Promega) according to the supplier's instructions, digested with *Eco*RI and subcloned in the low copy plasmid pWKS30 (Amp^r) (Wang & Kushner, 1991) giving plasmid p*Ec1arcA*.

Sensitivity to TB

To test the sensitivity to TB, cultures inoculated at an $OD_{580\,nm} = 0.05$ were grown overnight at 37 °C in LB supplemented with 100 μg mL^{-1} ampicillin when necessary. The cultures were diluted in saline, and 5 μL of appropriate dilutions were spotted on LB agar supplemented with 200 μgmL^{-1} (TB plates) and on LB plates. TB plates supplemented with 1% xylose and 1 mM IPTG were used for those strains capable of poly(3-hydroxybutyrate) accumulation. Appearance of colonies was analyzed after 24 h of incubation at 37 °C.

Table 1. *Escherichia coli* strains

Strain	Relevant characteristics	Reference
K1060*	F^- *fadE62 lacI*60 *tyrT58*(AS) *fabB5 mel*-1 *supF58*	Overath *et al.* (1970)
DH5α	ϕ80d *lacZ* ΔM15 *recA endA1* Δ(*lacZYA-argF*)*U169 deoR gyrA96 thi-1 hsdR17 supE44 relA1*	Gibco-BRL (Carlsbad, CA)
SP314*	Δ(*galK-bioD*)*76 relA*1 *spoT1 thi-1 deoC7* Δ(*deoD-arcA*)*253*	Roeder & Somerville (1979)
ECL547	Sdh^+ Φ(*sdh-lac*)	Iuchi & Lin (1988)

*Obtained through the *E. coli* Genetic Stock Center.

Additional analytical methods

Oxygen consumption was measured with a Clark type polarographic electrode (Biological O_2 Monitor, Model 53, Yellow Springs Instruments Inc., Yellow Springs, OH). The assay mixture containing 1% xylose in LB was equilibrated during 3 min at 37 °C, under magnetic stirring in an open measure chamber. After that, an aliquot of the culture was added to determine O_2 consumption and the measure chamber was immediately closed. The total volume in the measure chamber was 3 mL. Two minutes after starting the determinations, 30 μL of 100 mM KCN were injected to the chamber to study respiration inhibition, and 2 min later 50μL of TB 10 mg mL^{-1} were added. O_2 uptake was expressed as the variation of percentage of O_2 saturation per minute per milligram of protein.

Protein concentration was determined by the Folin phenol reagent method (Lowry et al., 1951) using bovine serum albumin as standard.

Reactive O_2 species generation was analyzed by measuring intracellular deacetylation and oxidation of 2,7′-dichlorodihydrofluorescein diacetate (DCDHF-DA, Sigma Chemical Co., St Louis, MO) (Somerville *et al.*, 2002; Balzan *et al.*, 2004) to the fluorescent compound 2,7′-dichlorofluorescein (DCF). DCDHF is highly reactive with H_2O_2. Overnight cultures of the appropriate strains (grown in LB-xylose 2% at 37 °C) were diluted 1 : 100 in the same medium supplemented, when required, with ampicillin and TB at 100 and 200 μg mL^{-1}, respectively. After growing for 4 h as indicated above, IPTG was added at 1 mM, and growth was continued 2 h further. DCDHF-DA (10 mM in absolute ethanol) was added to a final concentration of 50 μM. After incubating for two additional hours, cells were spun (9500 ***g***, 10 min, 4 °C), resuspended in cold HEPES (20 mM, pH 7.0), and placed on ice for 30 min. Two drops of $CHCl_3$ and one drop of 0.1% (w/v) sodium dodecyl sulfate were added, the cells were vortexed for 30 s (three times, with 1 min intervals on ice) and placed for 15 min on ice to allow 2,7′-dichlorofluorescein diffusion. Cell debris was pelleted and 500 μL of the supernatant were used to measure fluorescence in a spectrophotometer (Thermospectronic, AMINCO Bowman Series 2, Waltham, MA). The excitation and emission wavelengths were 490 and 521 nm, respectively. Relative fluorescence intensity was expressed as arbitrary units corresponding to 10^7 cells. Data presented are the mean of at least three determinations.

Results and discussion

Characterization of a *dye* deletion mutant

Several *arcA* mutants, with slightly different phenotypes, have been described in the literature. One of the best characterized of these mutants is strain SP314, that carries a deletion involving *arcA* and other genes (Roeder & Somerville, 1979). This mutant is sensitive to the dyes methylene blue and TB (the Dye phenotype).

In order to analyze whether the sensitivity of strain SP314 to the dyes was solely due to the absence of *arcA*, a complementation assay with p*Ec*1*arcA*, a plasmid containing *arcA*, was performed. Dye sensitivity of strain SP314 containing the plasmid vector, pWKS30, or the complementing plasmid was analyzed by observing colony phenotype in TB plates. The strain bearing the empty vector showed only residual growth at the lower dilution, while the strain containing the complementing plasmid was able to form colonies in this medium (Fig. 1a). Both strains gave rise to the same colony number and size when plated on LB agar (data not shown). These results indicate that the Dye phenotype of strain SP314, which cannot grow on medium containing TB, is due to the absence of *arcA*, as it could be complemented by a plasmid carrying only this gene.

TB diverts electrons to O_2

It has been proposed that the Dye phenotype observed in the *arc* mutants is due to an alteration in the electron transport chain that allows dyes to divert electrons in a futile flow (Lynch & Lin, 1996). Previous work showed that increased production of electron donors in *Escherichia coli arc* mutants results in an increased rate of O_2 consumption (Nystrom *et al.*, 1996). O_2 consumption was measured in stationary cultures of SP314 bearing either plasmid vector pWKS30 or plasmid p*Ec1arcA*. As expected, O_2

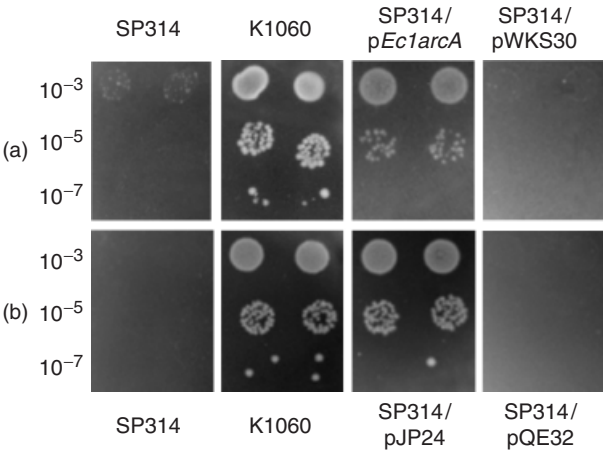

Fig. 1. Sensitivity to toluidine blue (TB) of different *Escherichia coli* strains. Cultures were grown for 18 h at 37 °C in Luria–Bertani (LB) medium (a) or LB supplemented with 1 mM isopropyl β-D-thiogalactosidase (IPTG) and 1% xylose (b). Strains carrying plasmids were grown in the presence of ampicillin 100 μg mL^{-1}. An aliquot (5 μL) of serial dilutions (10^{-3}, 10^{-5} and 10^{-7}) in saline were spotted in duplicate in TB plates (a) or TB plates supplemented with 1 mM IPTG and 1% xylose (b). Appearance of colonies was analyzed after incubation at 37 °C for 24 h.

consumption was twofold in the unregulated mutant carrying pWKS30 compared with the complemented strain (data not shown).

In order to investigate the possibility that TB captures electrons from the respiratory chain creating a nonenergy yielding flow towards O_2, the effect of TB on O_2 consumption was analyzed in the presence of cyanide, that blocks the last step of the transfer of electrons to O_2. Potassium cyanide (KCN) was used to inhibit the electron flow through the bacterial membrane, and TB was added to analyze its effect in restoring the electron flow. O_2 consumption was measured in stationary cultures of a control *arcA*+ strain (ECL547) and SP314. The addition of KCN caused a decrease in O_2 consumption that was relieved by TB, which restored O_2 consumption to the original levels (Fig. 2). These results indicate that TB is able to capture electrons flowing through the respiratory chain in a step before the one blocked by KCN, bypassing the electron flow blockage exerted by the respiratory inhibitor.

pha gene expression suppresses the dye sensitivity phenotype

If the inhibition of growth by TB in the unregulated *arcA* mutants is due to the futile electron transfer by the dye to O_2, then reducing the flow of electrons by sequestering a part of them in an electron sink such as PHA would suppress this phenotype. Growth of SP314 carrying either a plasmid that expresses the *pha* genes from *Azotobacter* sp. FA8, pJP24, or the empty vector, pQE32, on TB plates was analyzed (Fig. 1b). The *arcA* mutant with the vector did not form colonies on TB plates, whereas the strain carrying *pha* genes shows resistance to the dye, producing colonies similar to those produced by an *arcA*+ strain (K1060). All strains gave rise to the same colony number and size when plated on LB agar (data not shown). This experiment was repeated four times with the same results. As expected, the expression of the *Azotobacter* sp. FA8 *pha* genes suppressed the *dye* phenotype, allowing the *arcA* strain to grow in medium containing TB.

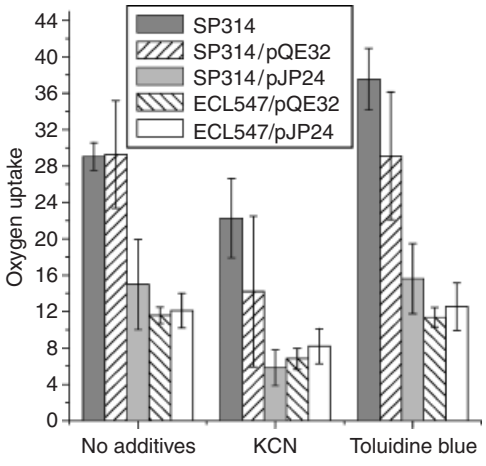

Fig. 2. Effect of potassium cyanide (KCN), toluidine blue and *pha* genes on O_2 consumption of *arcA* mutants. Cultures of *Escherichia coli* SP314, SP314/pQE32, SP314/pJP24, ECL547/pQE32 and ECL547/pJP24 were grown for 18 h in Luria–Bertani (LB) medium supplemented with 1% xylose and 1 mM isopropyl β-D-thiogalactosidase (IPTG). Ampicillin was added for those strains carrying plasmids. The figure shows O_2 uptake rates (expressed as the variation of percentage of O_2 saturation per minute per milligram of protein) measured in the absence of additives, after the addition of KCN and after the subsequent addition of toluidine blue. The experiment was repeated three times with similar results. The mean from triplicates of a representative experiment is shown.

pha gene expression decreases O_2 consumption in *arcA* mutants

Our work hypothesis was based on the role of poly(3-hydroxybutyrate) as an electron sink. According to this line of reasoning, poly(3-hydroxybutyrate) accumulation is expected to reduce the electron flow through the respiratory chain by capturing part of the electrons produced in the unregulated *arcA* mutants, thus decreasing O_2 consumption. O_2 consumption was measured in stationary cultures of a control *arcA*+ strain (ECL547) and SP314, either carrying a plasmid that expresses the *pha* genes from *Azotobacter* sp. FA8, pJP24, or the empty vector, pQE32. The strain carrying the *pha* genes showed an O_2 consumption rate about half of that observed for the mutant with the vector alone in all conditions, with values similar to those observed for the control *arcA*+ strain (Fig. 2). These results are in agreement with those obtained in TB plates, and indicate that poly(3-hydroxybutyrate) reduces the electron flow, thus decreasing O_2 consumption.

ROS

When cells grow aerobically, reactive by-products of O_2, such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals ($\cdot OH$), are generated. The majority of the ROS are produced as a result of reductions of molecular O_2 catalyzed by respiratory chain enzymes. These highly reactive O_2 species damage critical molecules, such as DNA, RNA, proteins and lipids (Cabiscol *et al.*, 2000).

On the basis of the observations made in mitochondria and in bacteria, it has been proposed that a faster rate of respiration hastens bacterial senescence and decay owing to a greater generation of ROS, that results in oxidative damage of macromolecules (Ballesteros *et al.*, 2001).

In *E. coli arcA* mutants, the cells are not able to regulate the production of electron donors according to the metabolic status of the cell, which results in an augmented rate of O_2 consumption. This could lead to the generation of an

increased amount of O_2 radicals, that the cells are unable to neutralize, resulting in oxidative damage.

As we observed that expression of *phb* genes in *arc* mutants lowers O_2 consumption, we measured the production of ROS in these mutants with and without *pha* genes to analyze if polymer synthesis diminished the production of these reactive species in the highly respiring *arcA* mutants. The production of ROS was determined for cultures of strain SP314, either carrying plasmid pJP24, or the empty vector, pQE32, with an without TB, as detailed in materials and methods. The fluorescence intensity values obtained in the absence of TB were of 8.36 ($\pm$0.94) and 2.99 ($\pm$0.64) for SP314/pQE32 and SP314/pJP24, respectively. The expression of *pha* genes significantly lowered ROS generation in the *arcA* mutant, indicating that the synthesis of poly (3-hydroxybutyrate) causes a decrease in the production of ROS. In addition, the amount of ROS was higher in the presence of TB, 15.62 ($\pm$1.85) and 6.79 ($\pm$0.72) for SP314/pQE32 and SP314/pJP24, respectively, showing a correlation between the increased O_2 consumption and a greater production of ROS in both strains.

Conclusions

In this work, we have demonstrated that sensitivity of *arc* mutants to TB, a previously unexplained phenotype, is due to an unregulated respiration rate, which results in higher O_2 consumption levels, increasing the production of reactive O_2 species that affect cellular viability by damaging macromolecules. On the other hand, the role of PHA as an electron sink in a heterologous background has been experimentally demonstrated, as the synthesis of this polymer was able to suppress dye sensitivity in *Escherichia coli arcA* mutants by decreasing electron flow through the respiratory chain, thus diminishing O_2 consumption and the production of damaging ROS.

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