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Activity of Catabolic Enzymes of Film-Forming Strains of *Staphylococcus aureus*

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Abstract. The activity of glucose catabolism pathways of film-forming strains of staphylococci isolated from vagina of women with dysbiosis of reproductive tract and from women without disorders of microflora was studied. It was established that the investigated film-forming strains utilized the carbohydrates by pentose phosphate pathway mainly, as indicated by 23-33% higher enzyme activity compare to strains isolated from healthy women. Instead strains, isolated from women without dysbiosis of reproductive tract, have higher activity of glycolytic enzymes on 13-28%. The prevalence of glycolytic transformation of glucose by strains isolated from healthy women also indicates by the depression of glucose oxidation during action of monoiodinacetate – classical inhibitor of glycolysis. It inhibits glycolysis of strains isolated from healthy women more significant. It was established that oxidase activity of film-forming strains isolated from women with dysbiosis, increased over 40% during the use of basic substrates of citric acid cycle. These data indicate a general increase of catabolic activity of oxidative type of staphylococci isolated during vaginal dysbiosis and able to form biofilm.

1. Introduction

A lot of research are devoted to study of reactions of energy and constructive metabolism of staphylococci. However, there are also a number of open issues, primarily related to the ratio of substrate's flow in glycolytic or pentose phosphate pathways. The one of open questions is the regulation of the load of catabolic processes and transition of the substrates from one cycle to another under different functional states of cells, during the influence of environmental factors, and also during the formation of microbial associations that colonize the open cavity of human body with formation of biofilms.

Methods of genomic and proteomic analysis that allowed to explain the regulation of structural genes of staphylococci responsible for the synthesis of certain enzymes and proteins provide significant progress in studied area [1-4]. However, it is necessary to make a systemic study of enzymes involved in metabolic processes to identify whole stages of metabolic transformations during the energy and constructive metabolism that provide vital functions of cells, including the expression of genes encode the factors of pathogenicity and virulence of microbes, and these studies should be carried out at different levels of structural organization: intact cells, cell-free homogenates, supramolecular complexes and intracellular organelles, isolated enzymes [5-9].

The aim of research was to investigate the intensity of metabolic processes of *Staphylococcus aureus* strains, able to form biofilm.

2. Materials and Methods

The study of metabolic processes was made with use of intact cells, cell-free homogenate fraction and membrane vesicles derived from the cultures of *Staphylococcus aureus* strains (n = 5), able to form biofilm and isolated from the vagina of women with reproductive tract dysbiosis. Control group consists from *S. aureus* strains (n = 5) isolated from vagina of women who had not disorders of the reproductive tract microflora.

Identification of selected strains based on signs listed in Bergey's manual of determinative bacteriology [10] with use of standard methods. Samples of biological materials were inoculated on the saline meat-peptonic agar (contents of NaCl 10%). Inoculated plates were incubated at 37 °C for 18-24 hours. Staphylococcal colonies were golden-yellow, circular and convex with entire margins, diameter – 2-4 mm. Material from colonies stained by Gram method. Staphylococci are Gram-positive cocci, which appears as grape-like clusters when viewed through a microscope (×900). Next step was study of bacteria on ability to use glucose and mannitol as single source of carbon in anaerobic condition with formation of acid and/or gas and on ability to product the coagulase [11-13]. If this test were positive strain indicate as *Staphylococcus aureus*.

The ability to form biofilm determined for pure culture of isolated strains that seeded in the wells of plate in an amount not less than 10^5 CFU / ml. The plates were incubated at 37 ºC for 72 h. If during this period film growth took place as settled on the walls film after culture medium removing, the strain considered as film-forming. For each strain 3 wells of plate were seeded. Results recognized as positive if at least in one of the three wells formation of biofilm took place within 72 h.

For cultivation of bacteria were used liquid or solid nutrient media. Culture of studied strains incubated at 37 °C until the end of the logarithmic growth phase. Then the cells were laundered twice by centrifugation at 5000 rpm 20 min in 50 mM tris(hydroxymethyl)-aminometan hydrochloride (pH 7.4) and suspended in the same buffer with the addition of 5 mM $MgCl_2$.

Cell-free homogenates received by ultrasound treatment of cells at 22 kHz for 15 minutes with an interval of 1 min at disintegrator UZDN-A (Ukraine). Indestructible cells were removed after centrifugation at 5000 rpm for 10 min.

Vesicle membrane fraction was obtained by differential centrifugation at 22 000 g 60 mins, the temperature was 3–4 °C.

For the study of catabolism intensity of staphylococci strains the dynamics of accumulation of end-products of metabolism, activity of key enzymes of major catabolic processes of staphylococci – glycolysis, pentose phosphate pathway, citric acid cycle were defined, also an inhibitor analysis was made.

The intensity of accumulation of lactate (main end-product of glucose utilization) by staphylococci determined during the dehydrogenation of lactate to pyruvate by restore of NAD to NADH [6].

Among glycolytic enzymes the activities of phosphofructokinaze, aldolase of fructose-1,6-bisphosphate and pyruvate kinase were determined. Determination is followed at 340 nm with Specord UV-VIS (DDR).

The phosphofructokinaze activity was determined in the presence of additional enzyme system containing fructose-bisphosphate aldolase, triosephosphate isomerase, glycerol-3-phosphate dehydrogenase by the decrease of reduced NADH [14].

The activity of aldolase of fructose-1,6-bisphosphate determined with additional enzyme system containing triosephosphate isomerase and glycerol-3-phosphate dehydrogenase by the decrease of reduced NADH [15].

Determination of pyruvate kinase made with adding of lactate dehydrogenase by the decrease of reduced NADH [5].

Study of activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and transketolase was made. The activity of glucose-6-phosphate dehydrogenase was carried out by the accumulation of reduced NADPH [16]. Activity of 6-phosphogluconate dehydrogenase was defined also by the reducing of NADP [17]. Determination of transketolase activity was made with used of additional enzyme system containing triosephosphate isomerase, glycerol-3-phosphate dehydrogenase and NADH by NADH consumption rate [18].

In all experimental series with addition or forming NADH during the different reaction to the incubation medium 1 mM of KCN was add to prevent the oxidation of NADH by enzymes of respiratory chain.

The intensity of oxidation of substrates in nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ of protein by intact cells and cell-free homogenates of staphylococci and influence of inhibitors were determined by polarographic method with use of Polarograph LP - 7 (Czech Republic).

Statistical analysis of the results was made using the t-Student test with significance level of 0.05.

3. Results and Discussion

As a criteria for assessment of the dynamics of catabolic processes of *S. aureus* strains were elected the intensity of accumulation of end-products of fermentation, activity of key enzymes of catabolic cycles, the intensity of substrate oxidation and inhibitor analysis with use of classical inhibitors of catabolic metabolism. In a number of studies found that staphylococci can degradate the carbohydrates, especially glucose, by glycolysis or glucosephosphate ways. As it known, the main end-product of staphylococci in glycolysis is lactate. Except it, in a much smaller quantity may be realized acetate and CO_2.

The intensity of lactate accumulation in aerobic and anaerobic conditions were determined (table 1).

Table 1. The intensity of lactate accumulation (nmol $min^{-1} \cdot mg^{-1}$ of protein) during aerobic and anaerobic conditions of cultivation of staphylococcal strains

Conditions / Groups	Aerobiosis nmol $min^{-1} \cdot mg^{-1}$	Anaerobiosis nmol $min^{-1} \cdot mg^{-1}$	Pasteur effect (%)
Control, n=5	65.0 ± 5.0	75.0 ± 4.0	13.3
Film-forming strais, n=5	85.0 ± 4.0*	105.0 ± 7.0*	19.0

Asterisks [*] denotes significant at P<0.05 between groups

Pasteur effect (PE), which is known to be one of the factors that regulate metabolism of bacteria during the transition from anaerobic to aerobic conditions relied on the formula:

$$PE = \frac{Q_{N_2} - Q_{O_2}}{Q_{N_2}} \cdot 100\%$$

where Q_{N_2} – the amount of lactate accumulated in anaerobic conditions; Q_{O_2} – the amount of lactate accumulated in aerobic conditions [6].

Pasteur effect of film-forming strains isolated from women with vaginal dysbiosis, was 19.0%, therefore the intensity of lactate accumulation is higher in anaerobic conditions. These values are typical for facultative anaerobic bacteria, that have not complete switch of metabolism during the transition from anaerobic to aerobic conditions [6].

Among glycolytic enzymes the activities of phosphofructokinaze, aldolase of fructose-1,6-bisphosphate and pyruvate kinase were determined. These enzymes are regulatory and central enzymes of glycolysis [4,5,15]. The activity of these enzymes is shown in table 2.

Table 2. The activity of glycolytic enzymes in the studied strains of staphylococci (nmol $min^{-1} \cdot mg^{-1}$ of protein)

Enzymes / Groups	Phosphofructokinaze	Aldolase of fructose-1,6-bisphosphate	Pyruvate kinase
Control, n=5	85.0±5.0	572.0±19.0	145.0±12.0
Film-forming strais, n=5	61.0±5.0*	496.0±25.0*	106.0±10.0*

Asterisks [*] denote significant at P<0.05 between groups

The activity of phosphofructokinaze in studied film-forming strains of staphylococci isolated during the dysbiosis, was 28.2% below the value of this enzyme in control group. The activity of aldolase of fructose-1,6-bisphosphate was below the benchmark on 13.3%. The activity of pyruvate kinase was lower by 26.9% compare to this enzyme of staphylococcal strains isolated from vagina of women without dysbiosis of reproductive tract. These data allow to provide the survival of the culture under influence of adverse factors of environment by reducing of the intensity of metabolic processes.

To expand the data about the intensity of glycolysis the experiments on the effects of a specific inhibitor of glycolysis – sodium monoiodineacetate (MIA) on the intensity of glucose oxidation of studied strains of staphylococci were made (table 3).

The experiments show that strains of staphylococci isolated during vaginal dysbiosis, characterized by increased oxidative activity compare to strains isolated from women without disabilities of vaginal microbiota. The addition of inhibitor MIA caused the inhibition of intensity of glucose oxidation: inhibitory effect was 20.0% for strains isolated during dysbiosis and 28.8% for strains isolated from healthy women. Received data may indicate that the main way of carbohydrates converting of staphylococci strains isolated at dysbiosis is a pentose phosphate pathway and for strains isolated from women without disorders of vaginal microbiota is a glycolytic way.

Table 3. Inhibition of intensity of glucose oxidation by MIA (10^{-3} mol) in intact cells of staphylococci

Additive / Groups	Glucose nmol $min^{-1} \cdot mg^{-1}$ of protein	Glucose+ MIA nmol $min^{-1} \cdot mg^{-1}$ of protein	The effectiveness of inhibition (%)
Control, n=5	590.0±54.0	420.0±36.0	28.8
Film-forming strais, n=5	640.0±61.0*	510.0±29.0*	20.0

Asterisks [*] denotes significant at P<0.05 between groups

It is known that glycolysis and pentose phosphate pathway are alternative ways to convert carbohydrates into catabolic metabolism of staphylococci. The activity of the pentose phosphate pathway was determined by study of activity of three enzymes: two of them are glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase relate to the oxidative part of the cycle, and transketolase belongs to the reducing part of the cycle [16-18]. Data about the activity of these enzymes are shown in table 4.

Table 4. The activity of pentose phosphate pathway enzymes of the studied strains of staphylococci (nmol $min^{-1} \cdot mg^{-1}$ of protein)

Enzymes / Groups	Glucose-6-phosphate dehydrogenase	6-phosphogluconate dehydrogenase	Transketolase
Control, n=5	165.0±16.0	155.0±18.0	95.0±9.0
Film-forming strais, n=5	220.0±19.0*	190.0±20.0*	120.0±14.0*

Asterisks [*] denotes significant at P<0.05 between groups

Analyzing the received data, we can note that the activity of these enzymes of strains isolated during dysbiosis, was higher than 23-33% compare with the results for strains isolated from women without reproductive tract dysbiosis. It confirms the assumption that strains isolated during dysbiosis characterized by increasing of activity of pentose phosphate pathway enzymes, especially of it oxidative part.

The next step of research of metabolic activity of staphylococci is determination of activity of certain enzymes of citric acid cycle (table 5).

Table 5. The intensity of citric acid cycle substrate oxidation (nmol $min^{-1} \cdot mg^{-1}$ of protein) of the studied strains of staphylococci

Substrate / Groups	Isocitrate	α-Ketoglutarate	Malate	Succinate
Control, n=5	85.0±7.0	75.0±9.0	255.0±23.0	190.0±23.0
Film-forming strais, n=5	120.0±12.0*	105.0±16.0*	360.0±27.0*	270.0±22.0*

Asterisks [*] denotes significant at $P<0.05$ between groups

The table presents data about the rate of absorption of oxygen in nmol O_2 converted for 1 minute to 1 mg of protein of suspension of staphylococci during the oxidation of substrates of citric acid cycle that under the relevant dehydrogenase action able to separate the protons, which come to respiratory chain of the staphylococcal cells [14]. These data indicate the presence of different dehydrogenases in both studied groups of staphylococci: isocitrate, α- ketoglutarate, malate and succinat. Comparing the data about the oxidase activity of studied strains of staphylococci isolated during vaginal dysbiosis with data that are found for strains isolated from women without vaginal dysbiosis, it will notice that they are characterized by raising oxidase activity more than 40% using studied substrates. The presented data about the oxidation of substrates of citric acid cycle correlated with data about increasing of activity of pentose phosphate pathway in converting of carbohydrates of studied strains of staphylococci isolated during vaginal dysbiosis. These facts indicate a general increase of oxidative type of catabolic activity among the film-forming strains of staphylococci isolated from women with vaginal dysbiosis.

4. Conclusion

1. It was found that Pasteur effect for film-forming strains of *S. aureus*, isolated during vaginal dysbiosis was 19.0%, and therefore the intensity of lactate accumulation is significant higher in anaerobic conditions.

2. It was shown that the activity of phosphofructokinaze, aldolase of fructose-1,6-bisphosphate and pyruvate kinase were lower on 13-28% compare with values of same enzymes for strains isolated from healthy women. The addition of MIA led to inhibition of intensity of glucose oxidation: inhibitory effect was 20.0% for the film-forming strains of staphylococci isolated during the dysbiosis and 28.8% for strains isolated from healthy women, that indicate a more important role of glycolysis in control group.

3. The increase of the activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and transketolase on 23-33% indicates the leading role of the pentose phosphate pathway in the transformation of carbohydrates of the film-forming strains of staphylococci isolated during vaginal dysbiosis.

4. The increase of activity of citric acid cycle substrate utilization more than on 40% compare to strains isolated from healthy women shows the overall increasing of catabolic activity of oxidative type among the film-forming strains of staphylococci isolated during vaginal dysbiosis.

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