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EXPERIMENTAL ARTICLES

Extracellular Factors Responsible for the Adaptation of *Pseudomonas fluorescens* Batch Cultures to Unfavorable Conditions

Yu. A. Nikolaev*, J. I. Prosser, and N. S. Panikov***

**Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117811 Russia*

***University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom*

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Abstract—The culture liquid filtrate of an exponential-phase *Pseudomonas fluorescens* batch culture added to another *P. fluorescens* culture at the moment of inoculation was found (1) to prevent or diminish cell adsorption of the flask walls, (2) to enhance the intensity of cell respiration, (3) to shorten the period of adaptation of LB-grown cells to growth in glucose-containing mineral M9 medium, (4) to stimulate bacterial growth at supraoptimum temperature (36°C) and pH values (4.8 and 9.2), and (5) to decrease the death rate of bacteria at the supraoptimum growth temperature. These results were interpreted as indicating that *P. fluorescens* cultures produce two types of regulatory exometabolites similar to those revealed earlier in *Escherichia coli* and *Bacillus subtilis* cultures: the direct-action adaptogenic factor X_I capable of increasing bacterial resistance to unfavorable growth conditions (temperature and pH) and factor of accelerated adaptation to new media. Both factors are presumably low-molecular-weight hydrophilic nonprotein compounds.

Key words: extracellular adaptogens, stress, new medium, adaptation, *Pseudomonas fluorescens*

Exometabolites (i.e., metabolites excreted into the medium) are very important to microorganisms for their normal growth. Some exometabolites, called pheromones, are involved in the regulation of various cell processes and can be used to control the quality of the environment [1, 2]. Many gram-negative bacteria communicate between each other through homoserine lactones (HSL), which are population density sensors [3] and are involved in the regulation of bioluminescence, antibiotic production, virulence, plasmid transfer, bacterial swarming, etc. Factor A of streptomycetes, γ-butyrolacton, controls secondary metabolism and cell differentiation [4]. *Micrococcus luteus* produces a polypeptide factor initiating the outgrowth of resting bacterial forms [5]. Factors d_2 of yeasts and bacteria, which represent unsaturated fatty acids, are involved in the regulation of autolysis [6]. Factors d_1 of yeasts and bacteria, which are acylated aromatic compounds, induce the formation of resting forms [7]. Numerous extracellular autoregulators control the population behavior and cytodifferentiation of myxobacteria [8].

Of great importance in protecting bacteria from unfavorable or conditions are the so-called protectants, such as betaine, ectoine, some amino acids [9], and exopolysaccharides [10], which are either present in the medium or synthesized by bacteria themselves. Stationary-phase marine vibrios produce compounds making them resistant to starvation and stresses [11]. The exponential-phase cells of *M. luteus* produce compounds stimulating the resuscitation of the dormant forms of this bacterium [5]. When exposed to hypoosmotic shock, *Halobacterium salinarium* excretes osmotic shock–signalling metabolites [15]. Thus, cell-to-cell communications in microbial populations are an interesting field of research, as is seen from increasing literature on this subject. Earlier, we described three extracellular factors synthesized and excreted by *Escherichia coli*: factor X_I capable of promoting adaptation to various stresses (exposure to antibiotics, *N*-ethylmaleimide (NEM), elevated temperature, and high NaCl concentrations) [12]; a factor of accelerated adaptation to new media (FAANM) [13]; and factor X_{II} capable of increasing cell viability in the presence of lethal NEM concentrations [13]. Analogous factors were also found in *Bacillus subtilis* [14]. In *Pseudomonas fluorescens* cultures, we detected some low-molecular-weight metabolites involved in the regulation of cell adhesion to the flask walls [18]. It should be noted that, in many cases, including all our works, extracellular adaptogenic factors were not isolated and characterized: they were described based only on the results of the action of culture liquids. Such an approach, as we think, is appropriate at the early stage of investigation of adaptogenic factors.

The aim of this work was to study the ability of *P. fluorescens* to produce, along with the adhesion autoregulators described earlier [18], extracellular factors

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of adaptation to stresses, analogous to those of *E. coli* (factors X_I, X_{II}, and FAANM) [12–14].

MATERIALS AND METHODS

The *Pseudomonas fluorescens* strain NCIMB 9046 used in this study was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) in Aberdeen (United Kingdom). The strain was grown at 30°C (unless otherwise specified) in 100- or 250-ml flasks with 10 or 50 ml of the medium on a Lab-line (United Kingdom) shaker (180 rpm). The growth medium, which was based on the M9 medium described in the textbook [16], contained 0.2% glucose; mineral components (g/l): Na_2HPO_4, 6.8; KH_2PO_4, 3; NaCl, 0.5; and NH_4Cl, 1; and microelements (mg/l): $MgSO_4 \cdot 7H_2O$, 247; $CaCl_2$, 14.7; $MnSO_4 \cdot 4H_2O$, 0.4; $ZnSO_4 \cdot 7H_2O$, 0.4; and $FeCl_3 \cdot 6H_2O$, 1 (pH 7.0) [17]. In some experiments, a rich medium LB [16] was used containing (g/l): tryptone (Oxoid), 10; yeast extract (Oxoid), 5; NaCl, 6. Material for inoculation was 16-h stationary-phase culture added in an amount of 2–5%.

Culture growth was monitored by measuring culture turbidity at λ = 600 nm, using a Pye-Unicam SP-450 UV/VIS spectrophotometer and a conversion coefficient of 270 μg protein/(ml OD_{600} = 1).

Experiments were carried out with exponential-phase cultures (3–4 h of growth) according to the protocol described earlier [14]. The amounts of biomass in control and experimental variants differed by no more than 10%.

The intensity of the energy metabolism of bacteria, which correlated with the intensity of their reproduction, was evaluated by the rate of CO_2 production in 10-ml cultures grown in 500-ml serum vials with screw caps. Culture turbidity was measured at 540 nm, using an SF-46 spectrophotometer (LOMO, Russia). The concentration of CO_2 in the gas phase was determined with the aid of an Infralit 4 IR analyzer (Junkalor Dessau, Germany).

Normal (unstressed) culture liquids (CLs) were prepared by filtering bacterial cultures (OD_{600} = 0.4–0.6) through 0.45-μm-pore-size filters (Millipore). The CLs of stressed cultures (hereinafter, stressed CLs) were prepared essentially in the same way except that, before filtration, the cultures were incubated at 37 or 43°C for 2 h or at 10°C 3 for 3 h. To elucidate the nature of putative adaptogenic factors, CLs were (1) incubated with 0.2 mg/ml proteinase K (BDH, United Kingdom) at 30°C for 2h; (2) passed through a Sep-Pak C_{18} reversed-phase column (Waters); and (3) passed through YM series ultrafiltration membranes (Millipore) with nominal molecular weight limits of 3000 and 10000 Da.

The biological action of CLs (stressed or unstressed) was evaluated by adding them to an exponential-phase *P. fluorescens* culture with OD_{600} = 0.2 (hereinafter, test culture) in a proportion of 1 : 1 (v/v)

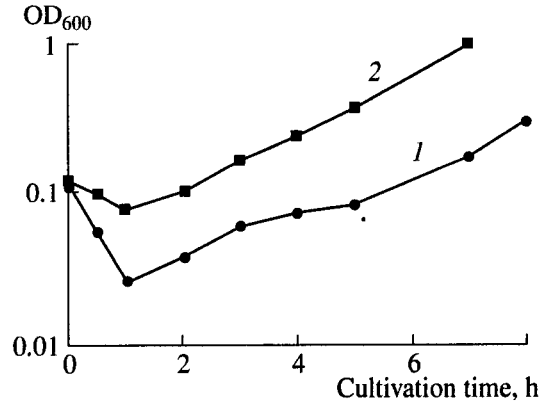

Fig. 1. Growth of *P. fluorescens* culture preliminarily grown in LB medium for 20 h after its mixing with (*1*) fresh M9 medium and (*2*) culture liquid.

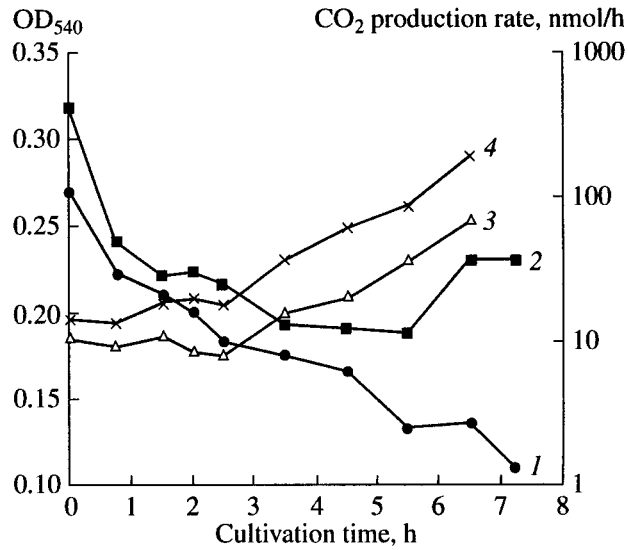

Fig. 2. Growth of *P. fluorescens* culture preliminarily grown in LB medium for 20 h after its mixing with (*1*, *3*) fresh M9 medium and (*2*, *4*) culture liquid: (*1*, *2*) OD_{540} and (*3*, *4*) CO_2 production.

(i.e., the turbidity of the test culture after mixing with the CL was 0.1). In the control experiments, the culture was mixed with an equal volume of fresh nutrient medium. The cultures thus prepared were incubated under either normal growth conditions (30°C; pH 7.0) or stress conditions (incubation at supraoptimum temperatures of 36 or 12°C, at supraoptimum pH values of 4.8 or 9.2, and in the presence of 30 or 200 μM NEM). In experiments to study the effect of pH, it was adjusted to desired values with KOH or HCl immediately after mixing the test culture with the CL tested or with a fresh nutrient medium.

Experiments were performed no fewer than three times. The results presented are the means of 2–3 replicated measurements in typical experiments. Statisti-

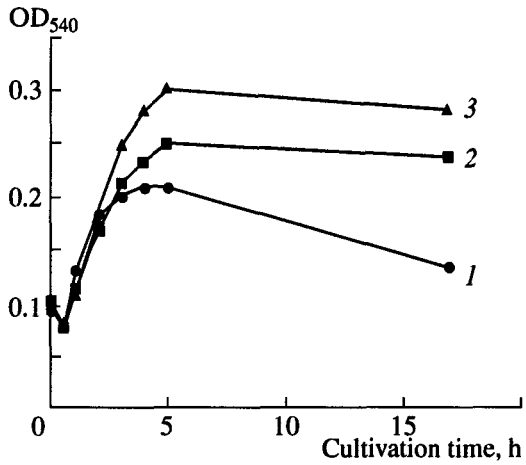

Fig. 3. Growth of *P. fluorescens* culture at 36°C after its mixing with (*1*) fresh medium, (*2*) unstressed CL, and (*3*) stressed CL.

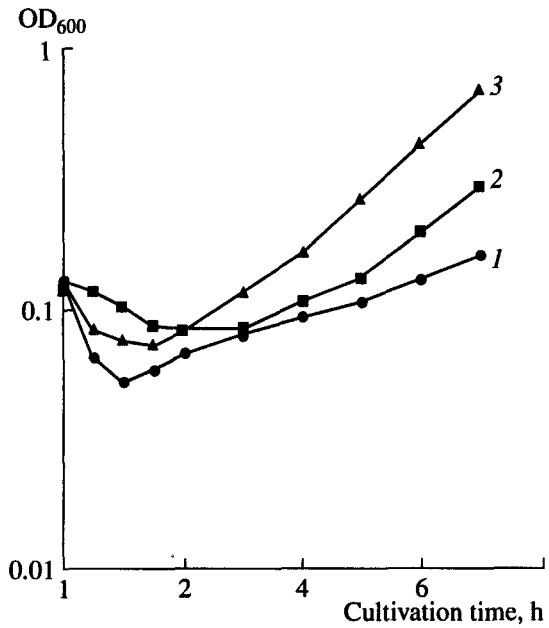

Fig. 4. Growth of *P. fluorescens* culture in the presence of 30 μm NEM after its mixing with (*1*) fresh medium, (*2*) unstressed CL, and (*3*) stressed CL.

cal parameters (arithmetic mean and standard deviation) were calculated using the Statgraphics software package.

RESULTS AND DISCUSSION

Our previous experiments, in which the exponential-phase *P. fluorescens* culture was transferred to a fresh nutrient medium with the normal growth temperature, showed that, during the first 1–1.5 h, the culture turbidity fell because of the cell adsorption to the flask walls [18]. Then, the cells adsorbed on the walls detached from them under the action of as yet unidentified autoregulator of adhesion (the so-called antiadhesin), and the culture resumed its exponential growth [18]. These observations should be taken into account when analyzing the results presented below.

P. fluorescens cells grown in LB broth to the logarithmic or stationary phase (6 and 20 h of growth, respectively) and transferred to the fresh M9 medium showed active growth after lag phases 1.5 ± 0.5 and 3 ± 0.5 h long, respectively (Fig. 1). The addition of the unstressed CL shortened the lag phases by 0.75 ± 0.5 and 1.5 ± 0.5 h, respectively. In both cases, the maximum growth rates were equal. These results suggest that the CL contains a factor promoting adaptation to new media (FAANM), such as the one described earlier for *E. coli* and *B. subtilis* [13, 14].

The effect of the unstressed CL on the adaptation of *P. fluorescens* culture to new media was also observed in those experiments in which the production of CO_2 was measured (Fig. 2). It should be noted that this parameter correlates well with other parameters characterizing the physiological state of cells, i.e., metabolic and growth rates and the change of growth phases [19]. Since CO_2 measurements were carried out using sealed flasks (this implies that cultures grew at lower oxygen concentrations than in the above experiments), the desorption of cells in these experiments began later (6–7 h after inoculation). In the control experiment, the CO_2 production rate within the first 2.5 h was low and virtually constant, indicating the absence of growth during the period of adaptation of the bacterial population to the new medium. When the CL was added to the culture, the CO_2 production rate was already 1.5–2 times higher in the first minutes of growth. Presumably, the increasing rate of CO_2 production during the next 2–3 h of cultivation in the presence of CL (in the control, the rate of CO_2 production did not rise) was due to increasing bacterial biomass. As is evident from Fig. 2, *P. fluorescens* showed some growth in the presence of CL within the first 2–3 h of cultivation, while growth was absent in the control. Two to three hours after inoculation, *P. fluorescens* began to grow with $\mu_{max} = 0.55\ h^{-1}$, irrespective of the addition of CL. It should be noted that this value of μ estimated from CO_2 production rate data agrees well with the value ($0.5\ h^{-1}$) estimated earlier from culture turbidity data [18]. Thus, the exponential-phase *P. fluorescens* culture liquid added to cells transferred to a fresh nutrient medium exerts the following effects:

(i) activates cell respiration (the contribution of glycolysis to CO_2 production in the case of sufficient aeration can be neglected);

(ii) stimulates culture growth suspended after transferring cells to a new medium;

(iii) promotes the "switching" of cell metabolism to another carbon source;

(iv) does not affect the maximum growth rate.

It should be emphasized that the experiment described confirms the inference that bacterial cells, when reversibly attached to the glass surface, continue to grow at the same specific growth rate as before attachment [18].

CL stimulated the growth of *P. fluorescens* cells taken from both exponential and stationary phases only when they were transferred from a nutritionally sufficient LB medium to a glucose-containing mineral M9 medium, while the stimulatory effect of the CL was not observed when the late-stationary-phase cells grown in M9 medium for 48 h were transferred to the same fresh medium. Namely, the 1- to 1.5-h lag phase observed in the control experiment did not shorten under the action of CL. Thus, CL affects the adaptation of metabolically active bacterial cells to new media but fails to activate metabolically inactive cells from the late stationary phase of growth. It can be inferred that the FAANM-induced shortening of the period of bacterial adaptation to nutritional changes is not associated with the effect of this factor on the physiological state of cells.

In the next set of experiments, we attempted to detect the direct-action adaptogen X_I and signal adaptogen X_{II} in *P. fluorescens* culture in the way these factors had been detected in *E. coli* and *B. subtilis* cultures.

Factor X_I protects bacterial cultures from various stresses. In preliminary experiments, we showed that temperatures of 36 and 12°C and pH values of 9.2 and 4.8, as well as the 30-μM concentration of NEM, were supraoptimum for the growth of *P. fluorescens* while more severe stress conditions led to growth cessation.

P. fluorescens culture transferred to the fresh medium and incubated at the elevated temperature of 36°C, continued to grow within 3–5 h (Fig. 3), after which the biomass gradually decreased. The addition of either stressed or unstressed CL to the *P. fluorescens* culture grown at 36°C lengthened the period of active growth by 1–3 h and increased the biomass by a factor of 1.5 to 2 (Fig. 3), while the rate of culture death in the post-stationary phase slightly decreased. The protective effect of the stressed CL was higher than that of the unstressed CL.

The alkylating agent of SH-groups and active oxidant, NEM, added to the medium simultaneously with the inoculum to a concentration of 30 μM caused a 1.5 ± 0.5-h lag period and about a twofold decrease in the maximum growth rate (from 0.48 ± 0.05 to 0.25 ± 0.05 h^{-1}), as well as led to an incomplete desorption of cells attached to the flask walls (Fig. 4). As shown earlier [18], the near-complete desorption of cells after their period of latent growth in the attached state is typical of *P. fluorescens* cultures grown under normal conditions. The incomplete desorption of *P. fluorescens* cells in the presence of NEM suggests that this compound suppressed the activity of antiadhesin. During the lag phase, *P. fluorescens* cells presumably inactivated NEM, resuming their exponential growth. Both stressed and unstressed CLs decreased cell adhesion due to the presence of antiadhesin [18] and enhanced the specific growth rate μ in the exponential phase from 0.25 ± 0.05 h^{-1} to, respectively, 0.35 ± 0.05 and 0.42 ± 0.05 h^{-1}.

Thus, under both oxidative (NEM-induced) and thermal stresses, CL promoted the adaptation of *P. fluorescens* cells. The activity of factor X_I in the stressed CL was higher than that in the unstressed CL. Unlike *E. coli* and *B. subtilis*, which synthesize extracellular X_I factor only under stress conditions [12], *P. fluorescens* can do the same under normal growth conditions.

In order to verify the suggestion that supraoptimum temperatures can induce the formation of factor X_I, we compared the adaptogenic activities of the CLs of 3-, 4-, and 5-h unstressed *P. fluorescens* cultures and the CL of the culture grown for 3 h under normal growth conditions and then for the next 2 h at the supraoptimum temperature of 37°C. It was found that the adaptogenic activity of CL (manifested as a rise in the specific growth rate within the first hours of growth, lengthening of the growth period by 1 to 3 h, and higher biomass yield) increased with the age of the culture from which a particular CL was prepared and was enhanced under the action of the supraoptimum temperature (Fig. 5). Therefore, factor X_I of *P. fluorescens* is synthesized in actively growing cultures, but supraoptimum conditions promote its biosynthesis.

When *P. fluorescens* was grown at pH 4.8 and 9.2, its specific growth rate was as low as 0.2 and 0.11 h^{-1}, respectively. Both unstressed and stressed CLs exerted a protective effect on the growth of *P. fluorescens* at these supraoptimum pH values: the specific growth rate at pH 4.8 and 9.2 increased to 0.33 and 0.17–0.2 h^{-1}, respectively.

At the same time, none of the CLs tested could promote the growth of *P. fluorescens* at 12°C, which occurred at a rate of 0.05 h^{-1}.

In order to elucidate whether factor X_I can be synthesized at low temperatures, the CL of *P. fluorescens* cultivated at 10°C for 3–4 h was tested for adaptogenic activity, using *P. fluorescens* cultures subjected to thermal and oxidative shocks. It was found that the adaptogenic activity of such CLs is higher than that of the unstressed CL and lower than that of the CL prepared from the thermally stressed culture (36°C). It is likely that cold stress basically differs from stresses induced by extreme pH values, high temperatures, freezing, toxic compounds, radiation, and other factors that inflict damage to subcellular structures, although cells respond to all these stresses in a similar way, i.e., by decreasing their growth rate. At low temperatures, metabolic processes are suppressed, while cellular structures are not damaged. In this case, bacterial cells presumably employ individual intercellular adaptation. In adaptation to thermal and oxidative shocks, causing structural and lethal damages, extracellular adaptogenic factors are employed. Correspondingly, the content of factor X_I in the cold CL was insignificant.

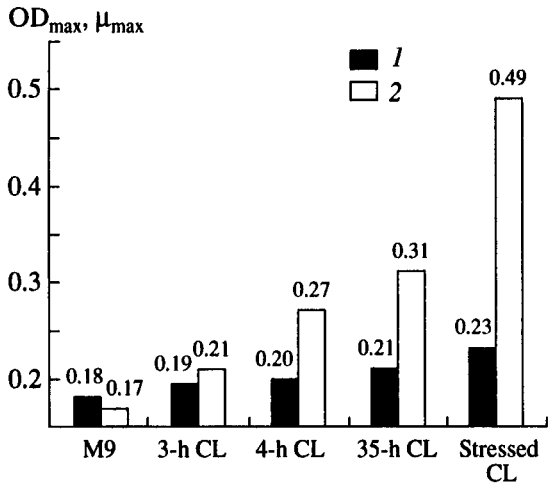

Fig. 5. Growth of *P. fluorescens* culture at 36°C after its mixing with fresh M9 medium and CLs prepared from the 3-, 4-, and 5-h unstressed cultures and 5-h stressed culture: (*1*) maximum culture turbidity, (*2*) specific growth rate within the first two hours of cultivation.

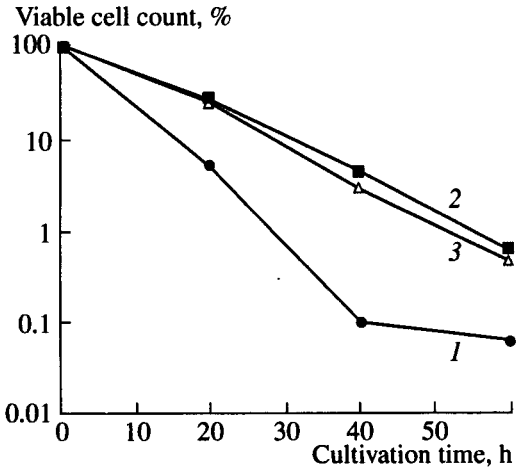

Fig. 6. Death rate of *P. fluorescens* culture in the presence of 200 μm NEM after its mixing with (*1*) fresh medium, (*2*) unstressed CL, and (*3*) stressed CL.

The ability of *P. fluorescens* to produce factor X_{II} was studied by elucidating the ability of its CL to suppress bacterial growth under normal conditions and to enhance cell viability under extremely unfavorable conditions, i.e., by examining the characteristics that are typical of the X_{II} of *E. coli* and *B. subtilis* [13, 14].

Experiments showed that the CLs of *P. fluorescens* cultures incubated at 37, 43, and 10°C did not suppress the growth of *P. fluorescens* under normal conditions. At the same time, such CLs increased the viability of *P. fluorescens* cells in the presence of 200 μM NEM (Fig. 6): the life time of cells in the presence of these CLs increased from 3 ± 1.2 to 6.6 ± 3 min. These results, however, can be explained by the protective effect of factor X_I and not by the presence of factor X_{II}.

CLs retained their adaptogenic activity after being subjected to ultrafiltration through membranes with a molecular weight limit of 3000 Da, passing through a reversed-phase Sep-Pak C_{18} column, and incubation with proteinase K. This suggests that factors X_I and FAANM are hydrophilic low-molecular-weight nonprotein substances. Moreover, FAANM is thermostable, as the heating of CLs at 100°C for 20 min did not destroy the activity assigned to this hypothetical factor.

Thus, *P. fluorescens* produces the extracellular direct-action adaptogenic factor X_I analogous to the one described earlier for *E. coli* and *B. subtilis* [12, 14]. Unlike the factor X_I of the two latter bacteria, which is synthesized only under extreme conditions, the factor X_I of *P. fluorescens* can be synthesized under normal growth conditions, although elevated temperatures stimulate its biosynthesis. The factor of accelerated adaptation to new media (FAANM) of *P. fluorescens* possesses properties similar to those of the FAANM of *E. coli*, and *B. subtilis*. *P. fluorescens* fails to synthesize adaptogenic X_{II} factor.

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