Establishment of *Staphylococcus warneri* in the Digestive Tract of Germ-Free Chicks and Isolation of the Bacterial Plasmid

Chieko HOSHINO, Yukiko SHIMIZU, Kunitomo WATANABE*,
Nobuhiro NIWA, Mitsuhiro FURUSE, Tatsuo MURAMATSU,
Junichi OKUMURA, Shoichi SHIMIZU and Kunio OHMIYA**

School of Agriculture, Nagoya University, Chikusa-ku, Nagoya-shi 464-01

* School of Medicine, Gifu University, Gifu-shi 500

** Faculty of Bioresources, Mie University, Tsu-shi 514

(Received August 27, 1990)

Abstract The anaerobic bacterium stimulating cellulose degradation of *R. albus* F-40 was identified as *Staphylococcus warneri* based on the results of gram-staining, fermentation tests and DNA-DNA hybridization. *S. warneri* was found to establish itself in the digestive tract of germ-free chicks but *R. albus* was not. One plasmid (7.7 kbp) denoted as pSW 1 was isolated from *S. warneri* and characterized.

Anim. Sci. Technol. (Jpn.) 62 (3) : 284-289, 1991

Key words : *S. warneri*, gnotobiotic chick, plasmid

According to a review written by MITSUOKA⁶⁾, some microflora inhabiting the digestive tract of chicks may accelerate the host growth and others may disturb it. There, the balance of both microflora should be very important to enhance the growth of chicks. This idea is derived from many studies on microflora in the digestive tract of chicks.

However, of these studies, only a couple of papers^{13,14)} describe the finding of cellulose-degrading microorganisms in the cecal of chicks, some of which digest cellulosic fiber in feed, but the resulting products are too small to be a sufficient energy source for the host chicks. Moreover, no study has ever proven how to improve the digestibilities of cellulosic materials via microorganisms which inhabit the chicks tract. In order to achieve this, we have to find or breed potent bacteria which can inhabit the chicks tract and improve the cellulose digesting ability of chicks. In this paper, we describe the establishment of

Staphylococcus warneri in the digestive tract of germ-free chicks and the isolation of a bacterial plasmid to improve the bacterial function genetically.

Materials and Methods

In order to identify the bacterium, the catalase test, Gram-staining test, fermentation test and DNA-DNA hybridization test were carried out. The catalase test was done by adding several colonies of the bacterium in 3% H₂O₂ solution. After Gram-staining, the morphology of the bacterium was observed by microscope. Fermentation properties of *Staphylococci* were determined with Apistaph and Apizyme (API SYSTEM S.A.). DNA hybridization test was performed with Southern blot analysis¹¹⁾ using biotin labelling reactions.

Two kinds of chromosomal DNA from *S. warneri* and *R. albus* F-40 were prepared via the method of SARTO and MIURA⁸⁾. Plasmid DNA were prepared via the method of

BIRNBOIM-Doly¹¹⁾:

A strictly anaerobic and cellulolytic bacterium *R. albus* F-40 was cultivated in an anaerobic medium containing yeast extract and ball-milled cellulose or cellobiose at 37°C for 19-43 h⁷⁾. A facultative anaerobe *S. warneri* was cultivated on trypticase soy broth (BBL Microbiology Systems) at 37°C for 19 h.

The separation and molecular weight evaluation of DNA longer than 1 kbp fragments were performed by electrophoresis with agarose gel (0.7-1.5%) using the method of SHARP *et al.*¹⁰⁾, and this electrophoretic analysis were done with polyacrylamide gel (5%) for DNA smaller than 1 kbp via the method of MANIATIS *et al.*⁵⁾. DNA fragments on the gel were detected with fluorescent light emitted by the short UV waves after ethidium bromide staining.

Southern blot analysis was conducted to identify the bacterium. For preparing the DNA probe, a biotin-labelling reaction was carried out using a nick translation kit (Takara) with the presence of biotin-7-dATP according to the protocol from the supplier. DNA fragments completely cleaved with restriction nucleases were separated by electrophoresis with 0.8% agarose slab gel and blot-transferred on the HybondTM-N membrane (Amersham), hybridized with the probe and detected subsequently by Blue GENETM for non radioactive nucleic acid detection.

For determining the plasmid copy number, the chromosomal and plasmid DNA extracted were quantitatively analyzed on an agarose gel electrophoretogram. The amount of each DNA on the electrophoretogram was measured with a scanning densitometer (DUAL-WAVE LENGTH TLC SCANNER CS-910, SHIMADZU) coupled to a microcomputer for peak integration. The molecular masses of chromosome were evaluated as approximately 2.6×10^9 daltons. The plasmid copy number (np) was calculated as follows ;

$$np = (Mc \cdot Cp) / Mp \cdot Cc$$

Mp and Mc were the molecular masses of the

plasmid and the chromosome, respectively, and Cp and Cc were the amount of plasmid and chromosomal DNA, respectively¹²⁾.

Single comb, White Leghorn chicks of mixed sexes were used, the parents of which (♂ : strain no. 09, ♀ : strain no. 18) were supplied by the from Gifu Prefectural Poultry Breeding Station in Japan. The experimental eggs were taken from the nest soon after laying to ensure freedom from fouling and clean eggs were selected for the production of germ-free chicks. The surface of these eggs were disinfected with formalin gas (potassium permanganate 7.1 g and formalin 14.3 g per cubic meter) for 10 min, thereafter, incubated in the disinfected incubator at 37.8°C for 18 days. Then, they were candled and disinfected again by spraying with peracetic acid solution (2%). The 18 days-incubated eggs were disinfected and introduced into four cages in each disinfected plastic isolators where they were incubated to hatch at 38°C. The humidity in the incubator was maintained at more than 70% until hatching. After hatching, one chick was left in each cage and others as well as egg shells were removed. Detailed procedures for hatching and maintaining germ-free chicks have been described elsewhere¹⁵⁾. The inoculum (1 ml, 10^9 cells/ml) of the mixture of *R. albus* and *Staphylococcus warneri* was poured with an injector having a polished needle into throat of 8 chicks of 3 days-age. Soon after inoculation, the diet disinfected by irradiation with ⁶⁰Co at 5 Mrad and sterilized water were freely taken by chicks. The ingredients of the diet granulated were chick mash (40%), soybean protein (17%), cellulose (28%), corn oil (9%), vitamins (1%), and mineral mixture (5%). The viable bacterial cells in feces produced by the chicks, after 1, 3, 7 and 18 days of bacterial inoculation, were detected using the media for both bacteria.

Sterility tests were undertaken at day 4 and day 12. Three liquid media {1. trypticase soy broth, 2. thioglycollate medium without indi-

cator-135 C (BBL, Cockeysville, Maryland, USA) and 3. sabouraud liquid medium (Oxoid Ltd, Basingstoke, Hants)} and two agar plates {1. tryptone soya agar (Oxoid Ltd) and 2. tryptone soya agar plus yeast extract (BBL)} were used¹⁵⁾. Liquid media were used to detect aerobes, and agar plates were used to determine both anaerobes and aerobes. The chicks were judged to be germ-free only when all these tests were negative for 2 weeks.

Results and Discussion

During the process of *R. albus* F-40 isolation, a facultative anaerobic bacterium was isolated with the medium for anaerobes containing glucose as a main carbon source. Cellulose degradation of *R. albus* F-40 increased significantly as shown in Fig. 1-C, in which the bulk of the cellulose in the medium was decreased markedly when the isolate was co-cultured with *R. albus* F-40. Therefore, we had great interest in an isolate which might be available to stimulate the cellulose-degrading ability of *R. albus*. The isolate did not solu-

bilize cellulose by itself but utilize glucose very well. Therefore, the stimulation phenomenon for cellulose degradation of *R. albus* by the isolate might be explained by that one strain can promote the growth of the counterpart by utilizing the fermentation products (2,4,9).

The organism was catalase positive and Gram staining positive. The morphological shape of the isolate was cocci similar to *R. albus*, making it difficult to distinguish them by shape. Fermentation tests and enzyme reaction tests suggested a classification of the bacterium as *Staphylococcus* sp. By employing DNA-DNA hybridization the bacterium was finally identified as *Staphylococcus warneri* with 100% relative homology to that of authentic *S. warneri*, judging from the intensity of radio isotope (data not shown). *S. warneri* have high esterase and lipase activities and have low β -glucosidase activity (data not shown).

The co-culture of *R. albus* and *S. warneri* was inoculated into germ-free chicks via mouth. Many viable bacterial cells were detected from chick faeces, both aerobically and anaerobically, from after two days of inoculation till the end of the experiment (18 days old), indicating that organisms inhabited the chicks. At day 18, 8 gnotobiotic chicks were killed by cervical dislocation and the digestive tract was soon separated into crop, duodenum, ileum, caecum and colon, from each of which the content was suspended into media for both organisms. Viable cells in each digestive tract were counted and shown in Table 1. The Gram positive cocci were found mainly in the caecum and colon and to a lesser extent in the other digestive tract (Table 1). From the DNA-DNA hybridization test, the bacteria inhabiting the chick was identified as *S. warneri*, not *R. albus* F-40 (Fig. 2), revealing that only *S. warneri* was established in germ-free chicks. When only *S. warneri* was inoculated into germ-free chicks, similar results to those shown in Table 1 were

Fig. 1. Bulk changes of ball milled cellulose in an anaerobic medium caused by *R. albus* and/or the isolate. A; Medium without inoculation. B; Culture of *R. albus* only. C; Co-culture of *R. albus* and an isolated strain. Arrows a, b, and c show the level of bulk of ball milled cellulose after cultivation of organisms.

Establishment of *S. Warneri* in Chick

Fig. 2. DNA-DNA hybridization between the isolate from gnotobiotic chick and *R. albus* or between the isolate and *S. warneri*

(A) ; Southern blot analysis (probe ; chromosomal DNA from *S. warneri*)
 (B) ; Southern blot analysis (probe ; chromosomal DNA from *R. albus*)
 (C) ; Agarose-gel electrophoretogram

Lane 1 ; Chromosomal DNA (20 μ g) from Gram-positive coccus digested with *Hind* III
 Lane 2 ; Chromosomal DNA (50 μ g) from *S. warneri* digested with *Hind* III
 Lane 3 ; Chromosomal DNA (20 μ g) from *R. albus* digested with *Hind* III
 Lane 4 ; Chromosomal DNA (80 μ g) from *R. albus* digested with *Hind* III
 Lane 5 ; λ DNA digested with *Hind* III

Table 1. Counts of microbial colony from the contents of crop, duodenum, ileum, caecum and colon of the gnotobiotic chicks

Gnotobiotic chicks	Crop	Duode-num	Ileum	Caecum	Colon
1	###	+	##	##	##
2	++	+	++	##	##
3	+	+	++	###	##
4	+	##	++	##	++
5	++	+	+	##	##
6	++	+	+	##	##
7	+	++	+	++	++
8	+	+	+	###	##

Gnotobiotic chicks were prepared from germ-free counterparts by inoculating *Ruminococcus albus* and *Staphylococcus warneri* at 18 days of age

+less than 100 colonies

++100~500 colonies

##500~1000 colonies

more than 1000 colonies

obtained, indicating that *S. warneri* was established in the digestive tract.

Via the modified SAITO-MIURA method, only one plasmid was isolated from *S. warneri*, the copy number of which was evaluated to be about 10 from Fig. 3. The size of the plasmid was 7.7 kbp and denoted as pSW 1. *Pst* I, *Eco*R I, *Pvu* II and *Hind* III sites were located on the plasmid as shown in Fig. 4. The existence of the single plasmid in the *S. warneri* strain showed very differences of the human and nonhuman primate *S. warneri* strain which often carry 2 to 10 plasmids³⁾. This simple plasmid system in *S. warneri* will help us to establish a novel host-vector system of *Staphylococcus* sp. Then it will be possible to confirm cellulolysis of *S. warneri* by a gene transfer system. These results have provided a clue toward improving the cellulose-diges-

Fig. 3. Agarose-gel electrophoretograms of plasmid and chromosomal DNA from *S. warneri*.

Lane 1; λ DNA digested with *Hind* III

Lane 2; Plasmid DNA from *S. warneri* digested with *Pst* I

Arrow indicates a plasmid from *S. warneri*

Fig. 4. Restriction map of pSW1 isolated from *S. warneri*

tibility of chicks, when the cellulase gene is integrated into the plasmid of *S. warneri* and expressed in a chick's digestive tract.

Acknowledgment

This work was supported in part by a grant-in aid for General Scientific Research C. No. 0156309 and Developmental Scientific Research (2) No. 63860038

References

- 1) BIRNBOIM, H.C. and J. DOLY, A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.*, **7**: 1513-1523. 1977.
- 2) DEHORITY, B.A. Hemicellulose degradation by rumen bacteria. *Federal Proceedings*, **32**: 1819-1825. 1973.
- 3) KLOOS, W.E., B.S. ORBAN and D.D. WALKER, Plasmid composition of *Staphylococcus* species. *Can. J. Microbiol.*, **27**: 271-278. 1980.
- 4) LATHAM, M.J. and M.J. WOLIN, Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of *Methanobacterium ruminantium*. *Applied and Environ. Microbiol.*, **34**: 297-301. 1977.
- 5) MANIATIS, T., E.F. FITCH and J. SAMBROOK, "Molecular Cloning", a laboratory manual. 173-178. Cold Spring Harbor Laboratory. New York. 1982.
- 6) MITSUOKA, T., Intestinal flora of chickens. *J. Jpn. Soc. Poultry Disease*, **13**: 113-127. 1977.
- 7) OHMIYA, K., K. NOKURA and S. SHIMIZU, Enhancement of cellulose degradation by *Ruminococcus albus* at high cellulose concentration. *J. Ferment. Technol.*, **61**: 25-30. 1983.
- 8) SAITO, H. and K. MIURA, Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta*, **72**: 616-629. 1963.
- 9) SCHEIFUIGER, C.C. and M.J. WOLIN, Propionate formation from cellulose and soluble sugars by combined cultures of *Bacteroides saccharigenes* and *Selenomonas ruminantium*. *Applied Microbiology*, **26**: 789-795. 1973.
- 10) SHARP, P.A., B. SUGDEN and J. SAMBROOK, Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using agarose-ethidium bromide electrophoresis. *Biochemistry*, **12**: 3055. 1982.

- 11) SOUTHERN, E.M., Detection of specific sequences among DNA fragments by gel electrophoresis. J. Mol. Biol., **98**: 503-517. 1975.
- 12) STEVEN, J.P., S. CARLETON and R.P. NOVICK, Determination of plasmid copy number by fluorescence densitometry. Plasmid., **9**: 182-190. 1983.
- 13) THORNBURN, C.C. and J.S. WILLCOX, The caeca of the domestic fowl and digestion of the crude fiber complex. I. Digestibility trials with normal and caeectomised birds. Brit. Poult. Sci., **6**: 23-31. 1965.
- 14) THORNBURN, C.C. and J.S. WILLCOX, The caeca of the domestic fowl and digestion of the crude fiber complex. II Experiments *in vivo* with fistulated birds, and the artificial and isolated caecum *in vitro*. Brit. Poult. Sci., **6**: 33-43. 1965.
- 15) YOKOTA, H., M. FURUSE, J. OKUMURA and I. TASAKI, A simple method for production and rearing of the germ-free chick. Jpn. J. Zootech. Sci., **55**: 600-603. 1984.

Staphylococcus warneri の無菌鶏ヒナ消化管への 定着とそのプラスミドの単離

星野智恵子・清水由希子・渡辺邦友*・丹羽信裕・古瀬充宏
村松達夫・奥村純市・清水祥一・大宮邦雄**

名古屋大学農学部, 名古屋市千種区 464-01

* 岐阜大学医学部, 岐阜市 500

** 三重大学生物資源学部, 津市 514

嫌気条件下でグルコースを資化するグラム陽性の通性嫌気性球菌の一種は, セルロース資化能を全く持たないが *R. albus* と混合培養すると *R. albus* のセルロース分解能を高めた. そこで分離菌株のグラム染色, 各種の糖の発酵能, および各種酵素活性を調べた結果, *Staphylococcus* sp. であることが判明した. さらに本菌の DNA を近縁株約 30 種の DNA とハイブリダイゼーションを行ない, *S. warneri* と同定した. 本菌の培養液を *R. albus* の培養液とともに無菌のヒナに 1 回だけ経口注入したところ, 2 日目より実験期間中常に *S. warneri* のみがフン中に検出された. 18 日齢になった時に全ヒナ腸管を摘出し, 内容物中の生菌を調べたところ, 盲腸と結腸に *S. warneri* が著しく定着していた. しかし, *R. albus* は全く検出できなかった. この結果, *R. albus* はヒナに定着しないが, *S. warneri* は定着することが判明した. 本菌のプラスミドを Birnboim-Doly 法により検索したところ, コピー数が約 10 で *Pst* I, *Eco*R I, *Hind* III, *Pvu* II の制限サイトをもつ 7.7 kbp のプラスミドの存在を確認し, これを pSW 1 と命名した.

日畜会報, 62 (3): 284-289, 1991