別紙4　　研究成果の刊行に関する一覧表

平成14年度

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
竹内 勤	血中赤痢アメーバ抗体価（間接蛍光抗体法）	和田 攻、他	臨床検査ガイド 2003~2004	文光堂	東京	2003	907~911
竹内 勤	アメーバ症、他		薬科微生物学	丸善	東京	2003	142~143

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fukao, T, Yamada T, Tanabe M, Ota T, Takayama T, Asano T, Takeuchi T, Kadowaki T, Hata J & Koyasu S	Selective loss of gastrointestinal mast cells and impaired immunity in PI3K-deficient mice	Nature Immunol	3	295~304	2002
Haghighi A, Kobayashi S, Takeuchi T, Masuda G & Nozaki T	Remarkable genetic polymorphism among Entamoeba histolytica isolates from a limited geographic are	J Clin Microbiol	40	4081~4090	2002
Makioka A, Kumagai M, Kobayashi S & Takeuchi T	Possible role of calcium ion, calcium channel and calmodulin in excytation and metacystic devleopment of Entamoeba invadens	Parasitol Res	88	837~843	2002
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竹内　勤	原虫感染症の最近の動向	臨床病理レビュー	121	156~ 161	2003
野崎智義、竹内　勤	寄生性原虫における硫黄含有アミノ酸生合成・分解経路－新しい抗原虫感染症薬剤開発標的	蛋白質核酸酵素	47	21~29	2002
野崎智義	アメーバ症	小児科診療	65	2132~ 2135	2002

平成15年度

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著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
竹内 勤	原虫性疾患－赤痢アメーバ症	杉本恒明、小俣政男、水野美邦	内科学、第8版	朝倉書店	東京	2003	438~440
竹内 勤	赤痢アメーバ症	黒川 清、松沢佑次	内科学、第2版	文光堂	東京	2003	2080~2082
竹内 勤、今井栄子、小林正規	寄生虫の院内(施設内)感染対策	小林寛伊、吉倉 廣、荒川宣親	エビデンスに基づいた感染制御－基礎編、改訂2版	メジカルフレンド社	東京	2003	144~152
野崎智義	アメーバ赤痢	神山恒夫、山田章義	動物由来感染症：その診断と対策	真興交易	東京	2003	244~249

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Dvorak JA, Kobayashi S, Nozaki T, Takeuchi T, Matsubara C	Induction of permebaility changes and death of vertebrate cells is modulated by virulence of Entamoeba spp.	Parasitol Int	52	169~173	2003
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Tachibana H, Watanabe K, Cheng X-J, Tsukamoto H, Kaneda Y, Takeuchi T, Ihara S, Petri WA Jr	VH3 gene usage in neutralizing human antibodies specific for Entamoeba histolytica Gal/GalNAc lectin heavy subunit	Infect Immun	71	4313~4319	2003

Makioka A, Kumagai M, Kobayashi S, Takeuchi T	Entamoeba invadens; inhibition of excystation and metacystic development by aphidicolin	Exp Parasitol	103	61~67	2003
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Ali V, Shigeta Y, Nozaki T	Molecular and structural characterization of NADPH-dependent D-glycerate dehydrogenase from the enteric parasitic protist Entamoeba histolytica	Biochem J	375	729~736	2003
Haghighi A, Kobayashi S, Takeuchi T, Thammapalerd N, Nozaki T	Geographic diversity among genotypes of Entamoeba histolytica field isolates	J Clin Microbiol	41	3748~3756	2003
竹内 勤	赤痢アメーバ症	総合臨床	52(増)	989~994	2003
小林正規、今井栄子、竹内 勤、野崎智義、Haghighi A	わが国における施設内赤痢アメーバ症の現況と問題点	微生物検出情報	24	81~82	2003
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著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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竹内 勤	血中赤痢アメーバ抗体価	和田 攻、大久保昭行、矢崎義雄、他	臨床検査ガイド	文光堂	東京	2005	813~817
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Beg MA, Kobayashi S, Hussainy A, Hamada A, Okuzawa E, Smego RA Jr, Hussain R, Takeuchi T	An experimental model for amebic abscess production in the cheek pouch of the Syrian golden hamster, Mesocricetus auratus	Parasitol Int	53	247~254	2004
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Ali V, Shigeta Y, Tokumoto U, Takahashi Y, Nozaki T	An intestinal parasitic protist Entamoeba histolytica possesses a ron-redundant NIF-like system for iron-sulfur cluster assembly under anaerobic condition	J Biol Chem	279	16863~16874	2004
Ali V, Shigeta Y, Hashimoto T, Nozaki T	Molecular and biochemical characterization of D-phosphoglycerate dehydrogenase from Entamoeba histolytica: a unique protozoan parasite that possesses both phosphorylated and non-phosphorylated serine metabolic pathways	Eur J Biochem	271	2670~2681	2004
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Remarkable Genetic Polymorphism among *Entamoeba histolytica* Isolates from a Limited Geographic Area

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In order to understand genetic polymorphisms among *Entamoeba histolytica* strains in a limited geographic area and among restricted social populations, we studied nucleotide polymorphism in DNA regions that do not encode proteins (locus 1-2 and locus 5-6) and in genes coding for chitinase and for serine-rich *E. histolytica* protein. Thirty *E. histolytica* isolates from domestically infected Japanese amebiasis patients (male homosexuals and residents in institutions for the mentally handicapped) and four reference strains were examined. PCR revealed remarkable polymorphisms in both the number and size of the PCR fragments containing these loci. Polymorphisms in lengths, types, and numbers of internal repeat units were observed in locus 1-2 and the repeat-containing region of serine-rich *E. histolytica* protein among the Japanese isolates. In contrast, polymorphism at locus 5-6 was observed almost exclusively in the number of repeats of a 16-nucleotide unit. The repeat-containing region of chitinase appeared to be the least polymorphic among the four loci with a single dominant genotype representing 66% (20 out of 30) of all of the isolates. Isolates obtained from male homosexuals showed a more complex genetic polymorphism than those from residents in institutions. Considering all four polymorphic loci together, all 19 Japanese isolates from male homosexuals were distinct. In contrast, all isolates obtained from mass-infection cases at a single institution had an identical genotype, suggesting that these cases were caused by a single *E. histolytica* strain. No significant correlation was found between genotypes and zymodemes or between genotypes and clinical presentations, e.g., colitis or liver abscess. Certain genotypes were observed with higher frequencies in male homosexuals or residents of institutions. These data indicate that genotyping of the *E. histolytica* isolates by using these four polymorphic loci could serve as a tool to fingerprint individual isolates. We propose that genotyping of ameba isolates should help to determine geographic origins of isolates and routes of transmission.

The protozoan parasite *Entamoeba histolytica* causes an estimated 50 million cases of amebiasis and 40,000 to 100,000 deaths annually, placing it second only to malaria as a cause of death resulting from parasitic protozoa (33). Since the first description of amebiasis in 1878 by Lösch (17), we still do not have a proper answer to the question of why disease and symptoms develop in only 5 to 10% of those infected with *E. histolytica*. It has been speculated that a spectrum of virulence levels among the *E. histolytica* strains and variability in the host immune response against amebic invasion contribute to the outcome of amebic infection. While variation in human immune responses against amebic infection is not understood, the polymorphic structure of *E. histolytica* has recently been unveiled (4, 7, 12, 28, 34). These studies have identified and characterized polymorphic DNA loci, including protein-coding sequences, such as those for the serine-rich *E. histolytica* protein (SREHP) (18) (also described as K2 [16]) and chitinase (12), as well as non-protein-coding regions, including the rRNA genes (4, 28), a strain-specific transcript (6), and loci 1-2 and 5-6 (34). These polymorphic loci have been shown to be potentially useful in investigating the molecular epidemiology of amebiasis. Ghosh et al. (12) and Zaki and Clark (34) showed significant polymorphism among *E. histolytica* isolates collected from a wide geographic range, e.g., Mexico, Bangladesh, India, Venezuela, and South Africa. However, whether or not genetic polymorphism also exists in an *E. histolytica* population in a restricted geographic location and, if it does, how extensive the polymorphism is remain unknown.

In contrast to the situation in countries where amebiasis is endemic, where transmission of amebas frequently takes place across wide geographic areas and social populations, amebiasis in Japan is prevalent only in limited social populations, i.e., male homosexuals (21, 22, 32) and institutionalized people, such as residents of institutions for the mentally handicapped (1, 14, 20). The high prevalence of *E. histolytica* infections in male homosexuals is a unique characteristic of amebiasis in Japan and is in sharp contrast to the dominant *Entamoeba dispar* infections in male homosexuals in western countries (2, 13, 27). In addition, Japan is one of a few developed countries where numerous *E. histolytica* isolates are identified in autochthonous amebiasis cases. To advance our understanding of the significance of the polymorphic population structure of *E. histolytica*, we have analyzed four polymorphic genetic loci of *E. histolytica* isolates obtained from domestically infected Japanese amebiasis patients. Based on the analysis of these loci, *E.*

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TABLE 1. Background and genotypes of the *E. histolytica* isolates

No.	Isolate	Source[a]	Isolation		Clinical diagnosis	Serology[c]	Zymodeme	DNA origin	Type			
			Location[b]	Date					Locus 1–2	Locus 5–6	Chitinase	SREHP
1	KU 1	H	Hospital 1	July 1994	Colitis	+	II	Xenic	B	A7	F	Mix[d]
2	KU 2	H	Hospital 2	December 1988	Colitis	+	XIX	Axenic	B	A7	C	Mix
3	KU 3	H	Hospital 3	September 1988	Colitis	–	II	Axenic	B	A6/Cv	A	M
4	KU 4	H	Hospital 4	July 1994	NA[e]	+	XIX	Xenic	B	A8	C	F
5	KU 5	H	Hospital 5	November 1988	Asymptomatic	+	II	Axenic	B	A6/Cv	A	M
6	KU 7	H	Hospital 6	March 1995	Colitis, ALA[f]	+	II	Xenic	B	A8	C	Mix
7	KU 8	H	Hospital 6	May 1995	Colitis, ALA	+	XIV	Xenic	E	A10/A5	B	Mix
8	KU 9	H	Hospital 6	May 1995	NA	+	II	Xenic	B	A7	C	G/M
9	KU 10	H	Hospital 4	October 1991	Colitis	+	II	Xenic	B	A8/Cv	A/C[g]	Mix
10	KU 11	H	Hospital 6	February 1994	ALA	+	XIV	Xenic	E	A5	C	B/L
11	KU 15	H	Hospital 6	September 1994	Colitis	+	XIX	Xenic	E	A5v	B	E
12	KU 16	H	Hospital 4	May 1993	Colitis	+	XIX	Xenic	D	A5v	C	Mix
13	KU 23	H	Hospital 7	February 2000	Colitis	+	XIX	Xenic	D	A13/A5	B	F
14	KU 30	H	Hospital 8	March 2001	Asymptomatic	+	II	Xenic	G	A5v	B	E
15	KU 31	H	Hospital 8	March 2001	Asymptomatic	+	II	Xenic	G	A5	C	E
16	KU 32	H	Hospital 9	June 2001	Colitis	+	XIV	Xenic	G	A5	B	E
17	KH 5	H	Hospital 6	2001	Colitis, HIV positive	+	ND[h]	Stool	G	A5v	C	I
18	KH 9	H	Hospital 6	2001	Colitis, HIV positive	ND	ND	Stool	B	A8	ND	ND
19	KH 15	H	Hospital 6	2001	ALA, HIV positive	+	ND	ALA	D	A6/Cv	D	ND
20	KU 13	M	Institution A	November 1999	Asymptomatic	+	XIX	Xenic	B	A7	C	H
21	KU 14	M	Institution A	November 1999	Asymptomatic	+	XIX	Xenic	B	A7	C	H
22	KU 18	M	Institution B	1994	NA	+	II	Xenic	F	A5v/Cv	C	K
23	KU 19	M	Institution B	1994	NA	+	II	Xenic	F	A5v/Cv	C	K
24	KU 20	M	Institution B	1994	NA	ND	II	Xenic	F	A5v/Cv	C	K
25	KU 21	M	Institution B	1994	NA	ND	II	Xenic	F	A5v/Cv	C	K
26	KU 22	M	Institution B	1994	NA	ND	II	Xenic	F	A5v/Cv	C	K
27	KU 26	M	Institution C	September 2000	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
28	KU 27	M	Institution D	January 2001	Asymptomatic	+	II	Xenic	C	A7	C	A
29	KU 28	M	Institution D	January 2001	Asymptomatic	+	II	Xenic	C	A7	C	A
30	KU 29	M	Institution D	January 2001	Asymptomatic	+	II	Xenic	C	A7	C	A
31	HM-1:IMSS cl6[i]	NA	Mexico	NA	Rectal ulcer, dysentery	NA	II	Axenic	D	A9/A6	C	O/P
32	SAW755[i]	NA	India	NA	Colitis	NA	XIV	Axenic	D	A6	C	C/N
33	SAW1627[i]	NA	India	1983	Asymptomatic	NA	IIα-	Axenic	A	A4/Cv	E	J
34	SAW1453[i]	NA	NA	NA	NA	NA	XIV	Xenic	A	A7	C	D

[a] H, homosexual male; M, *mentally* handicapped.
[b] Hospitals 1, 2, 4 to 9 are located in Tokyo; hospital 3 is located *in Kyoto;* institution A is located in Okayama prefecture; institution B is located in Kanagawa prefecture; and institution C and D are located in Shizuoka prefecture.
[c] Serology was done by enzyme-linked immunosorbent assay, gel diffusion precipitin test, and/or indirect fluorescent-antibody test.
[d] Mix, likely a mixture as judged by sequencing.
[e] NA, not available.
[f] ALA, amebic liver abscess.
[g] A mixed culture or heterozyogosity could not be ruled out (see text).
[h] ND, not determined.
[i] Reference strain.

histolytica isolates from Japan are very polymorphic, suggesting that *E. histolytica* has a complex clonal structure even in a limited geographic area and social populations.

MATERIALS AND METHODS

***E. histolytica* isolates and clinical samples.** A total of 34 *E. histolytica* samples were analyzed in this study. Twenty-seven Japanese *E. histolytica* isolates (Table 1) were obtained from clinical specimens collected from amebiasis patients in Japan. Xenic or axenic in vitro cultures were established and maintained in Robinson's medium or Diamond's BI-S-33 medium, respectively, as previously described (10, 25). Nineteen samples (samples 1 to 19) were obtained from male homosexuals, whereas 11 samples (samples 20 to 30) were from mentally handicapped individuals. The samples from the male homosexuals, who visited outpatient clinics, included those from 14 symptomatic cases, three asymptomatic cases, and two cases for which a history was not available. All isolates from mentally handicapped individuals were collected from four institutions geographically separated, i.e., Okayama (institution A), Kanagawa (institution B), and Shizuoka Prefecture (institutions C and D), within a week after mass infection was observed at institutions A to C at different time points (Table 1) (1994 to January 2001). No outbreak was observed at institution D. Most of the xenic and axenic strains were cryopreserved according to Diamond's method (9) immediately after xenic and axenic cultures were established and were revived 1 to 3 months prior to the present study to minimize possible changes, if any, of genotypes. Two *E. histolytica*-positive fecal samples and one liver aspirate were obtained from human immunodeficiency virus (HIV)-infected patients and kept frozen until DNA extraction. None of the donors had been abroad, and thus they are presumed to have been infected domestically. All cases with intestinal amebiasis or liver abscess were diagnosed by microscopic demonstration of trophozoites or cysts in stool or of trophozoites in liver aspirate, respectively. Past or present history of invasive amebiasis of these patients was verified with serology using the gel diffusion precipitin test (24) and enzyme-linked immunosorbent assay (31), and 25 out of 30 cases were considered invasive amebiasis. *E. histolytica* reference strains HM-1:IMSS cl6 (11), SAW755CR clB (8), SAW1627, and SAW1453 (5) and *E. dispar* strain SAW1734R cl AR (19) were used as controls. The *E. dispar* trophozoites were cultivated as previously described (15). All clinical specimens were collected after obtaining informed consent, and the research described in this paper complied with all relevant institutional guidelines.

DNA preparation and PCR analysis. Total genomic DNA from trophozoites and/or cysts was purified from either cultured amebas or clinical specimens by using the QIAamp DNA stool minikit (Qiagen, Tokyo, Japan) according to the

TABLE 2. Oligonucleotide primers

Primer name	Primer sequence (5′ to 3′)	Nucleotide position[a]	Accession no.
P11 (sense)	GGA GGA GTA GGA AAG TTG AC	214–234	D10512
P12 (antisense)	TTC TTG CAA TTC CTG CTT CGA	293–314	D10512
P13 (sense)	AGG AGG AGT AGG AAA ATT AGG	213–234	D00872
P14 (antisense)	TTC TTG AAA CTC CTG TTT CTA C	292–314	D00872
R1 (forward)	CTG GTT AGT ATC TTC GCC TGT	1–21	AF276055
R2 (reverse)	CTT ACA CCC CCA TTA ACA AT	383–401	AF276055
R5A (forward)	CTA AAG CCC CCT TCT TCT ATA ATT	1–24	AF276060
R6A (reverse)	CTC AGT CGG TAG AGC ATG GT	405–424	AF276060
Chitinase (sense)	GGA ACA CCA GGT AAA TGT ATA	466–487	U78319
Chitinase (antisense)	TCT GTA TTG TGC CCA ATT	799–817	U78319
SREHP (sense)	GCT AGT CCT GAA AAG CTT GAA GAA GCT G	258–286	M80910
SREHP (antisense)	GGA CTT GAT GCA GCA TCA AGG T	784–806	M80910

[a] Nucleotide positions to which the primer anneals.

manufacturer's directions. We determined DNA concentrations in samples by measuring optical absorbance at 260 and 280 nm spectrophotometrically. To verify that all cultures and samples contained only *E. histolytica* and not *E. dispar*, we amplified a 100-bp *E. histolytica*-specific fragment and a 101-bp *E. dispar*-specific fragment by PCR with a set of species-specific primers (P11 and P12 for *E. histolytica* and P13 and P14 for *E. dispar*) (Table 2) under the conditions described previously (30). We further classified the individual *E. histolytica* isolates by PCR amplification of four previously described polymorphic loci, i.e., locus 1-2, locus 5-6 (34), chitinase, and SREHP (12), using four sets of oligonucleotides previously described (Table 2). PCR was carried out in a 50-μl reaction mixture containing 0.1 μg of DNA, a 1.5 μM concentration of each primer, 2.5 mM $MgCl_2$, 0.1 μg of bovine serum albumin per μl, a 100 μM concentration of each deoxynucleoside triphosphate, and 1.5 U of HotStar *Taq* DNA polymerase (Qiagen) with the following cycling parameters: (i) *Taq* activation at 95°C for 15 min; (ii) 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C (SREHP and locus 5-6) or 45°C (chitinase and locus 1-2) for 30 s, and extension at 72°C for 1 min; and (iii) extension at 72°C for 10 min. PCR products were electrophoresed in 2% NuSieve 3:1 agarose (BioWhittaker Molecular Applications, Rockland, Maine). The results were visualized after staining with ethidium bromide in a UV transilluminator as described previously (26).

Sequence analysis. PCR products containing locus 1-2, locus 5-6, SREHP, and chitinase were directly sequenced with appropriate primers in both directions. All of the PCR samples that were found to contain single bands on the agarose gels were treated with a Pre-Sequencing kit (USB Corporation, Cleveland, Ohio) before sequencing. Each DNA fragment of the PCR samples that showed double or triple bands by agarose gel electrophoresis was excised and treated using a Geneclean II kit (BIO101, La Jolla, Calif.). Individual PCR products were then sequenced using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif.) on an ABI PRISM 310 Genetic Analyzer. The sequences obtained were manually edited and aligned using DNASIS (version 3.7; Hitachi, Yokohama, Japan).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in the present work have been submitted to the GenBank/EMBL/DDBJ database under accession numbers AB075701 to AB075737.

RESULTS

Polymorphisms in DNA patterns on agarose gels. The PCR fragments containing locus 1-2, locus 5-6, chitinase, and SREHP from the 30 Japanese strains and the four reference strains showed remarkable polymorphism in both the number of bands and their sizes (Fig. 1 [only representative isolates that belong to each genotype are shown]), as previously shown (7, 12, 34). The patterns and sizes of amplified fragments corresponding to locus 5-6 or SREHP were highly variable; these PCR fragments consisted of either single or double bands, which is consistent with these loci being either homo- or heterozygous (Fig. 1B and D). In contrast, apparently single PCR fragments corresponding to locus 1-2 and chitinase were observed in all of the isolates tested except for the one described below; only three or four groups among the isolates were distinguishable by the size of the bands (Fig. 1A and C). The presence of two chitinase bands in the KU10 isolate might indicate heterozygosity or a mixed culture (see Discussion). No isolates showed more than two amplified bands of locus 5-6, despite repeated PCR attempts under different conditions, which may indicate that the multiple (>2) bands previously observed (34) may be artifactual. Alternatively, this discrepancy may be due to different clones of HM1:IMSS used.

Polymorphism in nucleotide sequences of the noncoding DNA loci among the Japanese isolates. In order to better understand the nature of the polymorphisms among the Japanese strains observed by gel electrophoresis, we sequenced the individual fragments of locus 1-2, locus 5-6, chitinase, and SREHP from all 34 isolates. The nucleotide polymorphisms of these loci were more pronounced than those shown by gel electrophoresis. Although both locus 1-2 and locus 5-6 are present as tandemly linked multiple copies (34), individual DNA fragments in an apparently single band seemed to be homogeneous, suggesting that sequences in these copies are mostly identical. Both locus 1-2 and locus 5-6 contained 6 to 21 copies of 8- to 16-nucleotide repeat units, which is consistent with the previous findings (34). Sequencing of locus 1-2 revealed a complex interisolate polymorphism in length, location, and number of the repeat units (Fig. 2). Based on the nucleotide sequences of locus 1-2, the 30 Japanese isolates were divided into six distinct types (B to G), with a single genotype, B, being the dominant type (37%).

In contrast to the case for locus 1-2, PCR-amplified fragments of locus 5-6 were observed as either single or double bands depending upon the isolate, as mentioned above. Sequencing of the individual bands revealed that polymorphism among these bands was solely due to variations in the number of a 16-nucleotide repeat unit (GTATGTATATTTCTAT; 4 to 13 repeats), with a few exceptions (types A5v, B, C, and Cv). Individual bands of locus 5-6 were designated based primarily on either the presence or absence of the first GTATGTTTC TAT and the second GATTTTAT repeats (Fig. 3) (types A to C), second on the number of the GTATGTATATTTCTAT repeats (A4 to A13), and third on the presence or absence of nucleotide substitutions in the conserved region (A5v and Cv) (Fig. 3). As individual isolates appeared to be either homo- or

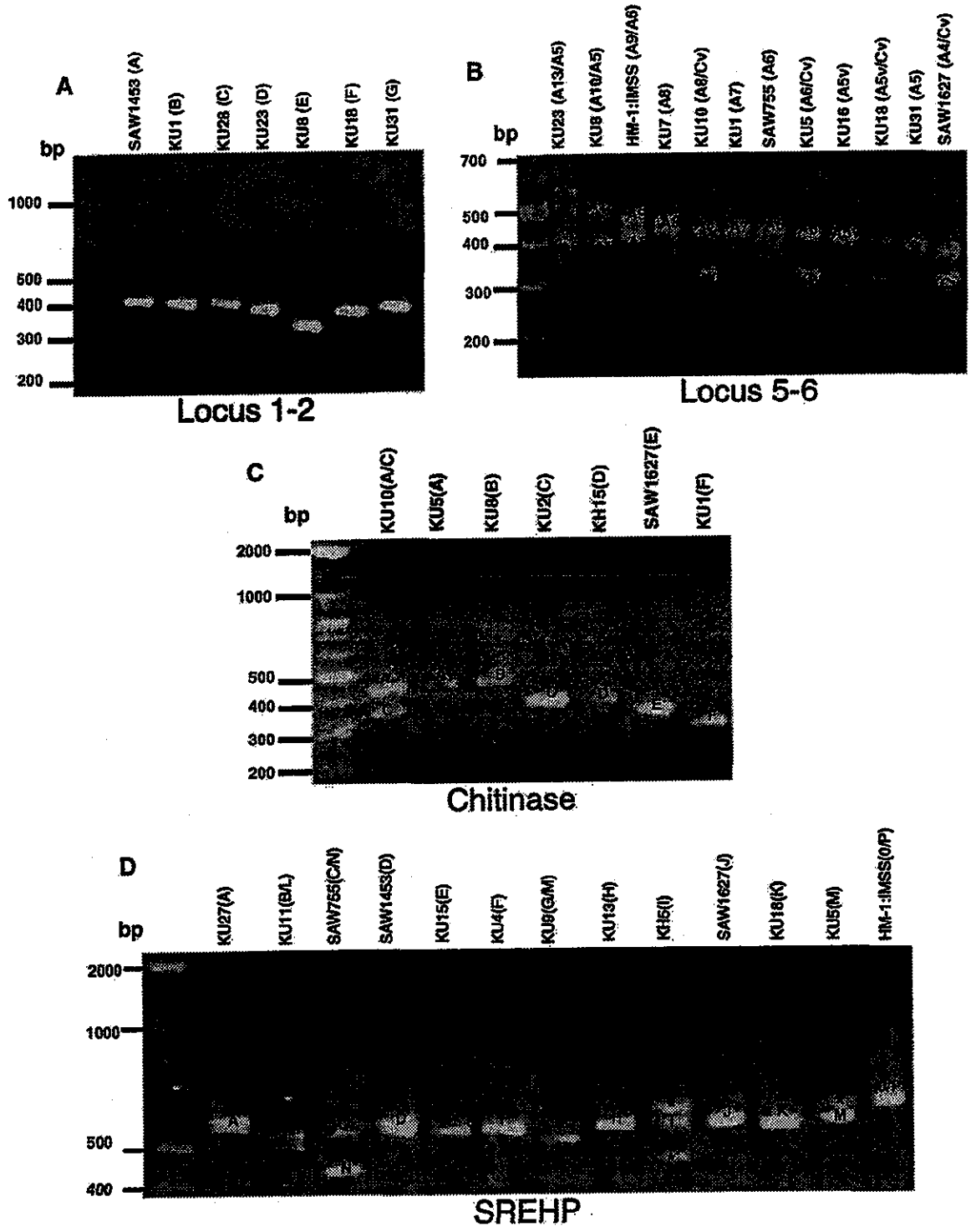

FIG. 1. Agarose gel electrophoresis of locus 1-2 (A), locus 5-6 (B), chitinase (C), and SREHP (D) from representative *E. histolytica* isolates. Only results for representative isolates that belong to each genotype (shown in parentheses) are shown. The individual genotype is designated for each polymorphic DNA fragment. Bands with asterisks are irrelevant PCR fragments (verified by sequencing) observed only in KU1, KU5, KH5, KH9, and KH15, for unknown reasons.

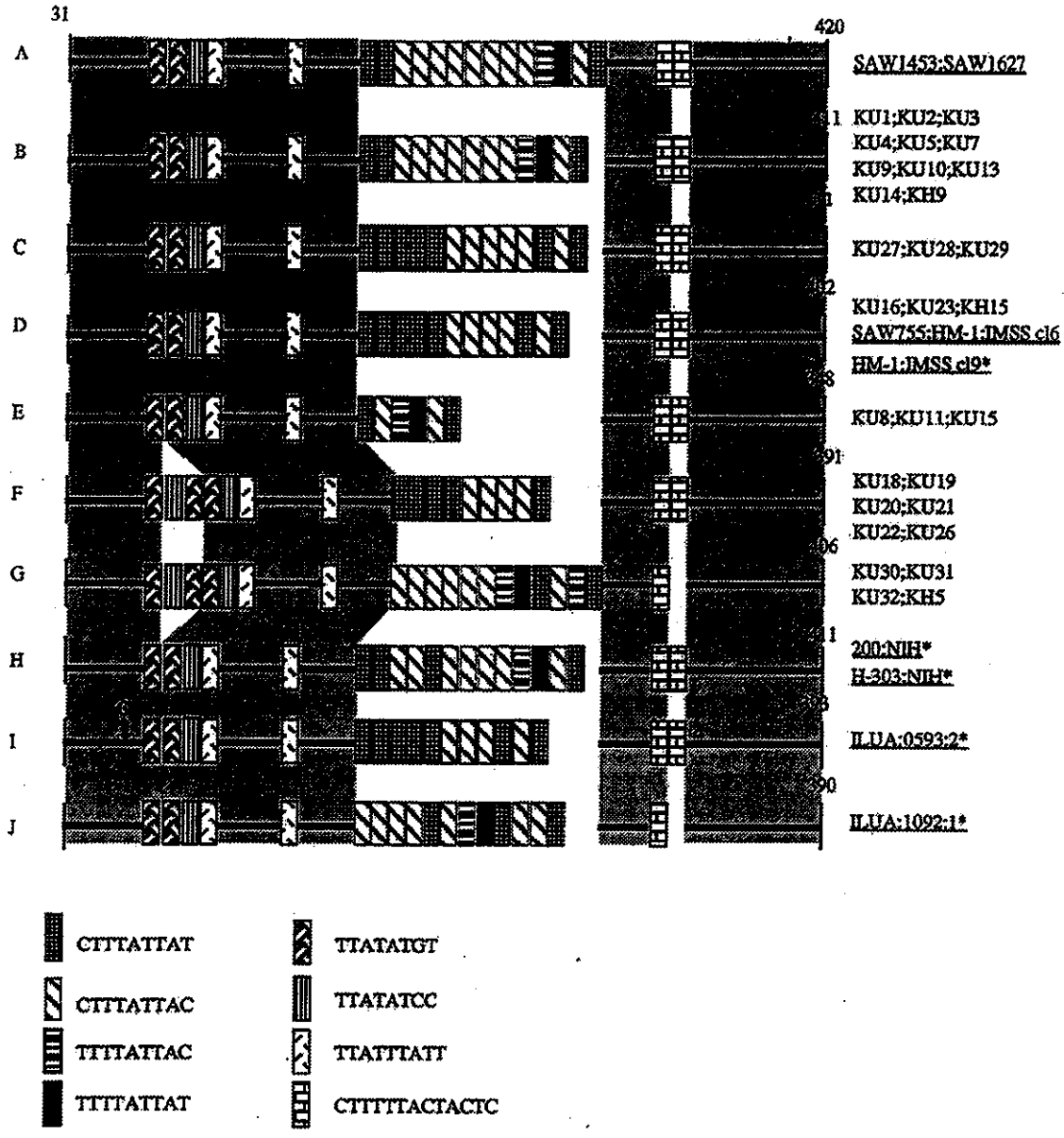

FIG. 2. Schematic representation of polymorphism in locus 1-2 among the Japanese isolates and reference strains. The numbers shown correspond to nucleotide numbers of locus 1-2 of HM-1:IMSS cl9 (GenBank/EMBL/DDBJ accession number AF276055). Sequences prior to nucleotide 30 are not shown because these nucleotides overlap the PCR primer. Gaps are introduced to optimize alignments. Conserved regions are highlighted with gray rectangles. Names of reference strains previously reported are underlined, and those analyzed in this study are double underlined. Previously unidentified repeats units are also included in this and following figures. A T-to-C nucleotide substitution is indicated by an arrow. An asterisk next to an isolate designation indicates that more information can be found in reference 34.

heterozygous at locus 5-6, we designated the genotype of each isolate, e.g., A7, A13/A5, and A5v/Cv.

Polymorphism in the chitinase and SREHP loci among the Japanese isolates. Polymorphisms in the type, location, and number of repeat units were observed in the repeat-containing region of the chitinase gene (Fig. 4). However, the chitinase locus appeared to be the least polymorphic among the four loci, with a single dominant genotype, C (out of five types observed in the Japanese isolates), comprising 66% (20 out of 30) of all of the Japanese isolates. All Japanese and reference isolates, as well as five reported sequences (12), were classified into only six independent types (Fig. 4).

The repeat-containing region of SREHP was found to be extensively polymorphic in size, species, number, and order of repeat units among the Japanese isolates (Fig. 5). Consistent with a previous notion that the SREHP locus is either homo- or heterozygous (12), either single or double bands of amplified SREHP fragments were observed, depending upon the isolate (see above). In addition, sequencing of gel-purified PCR bands of the SREHP loci from six isolates that showed an

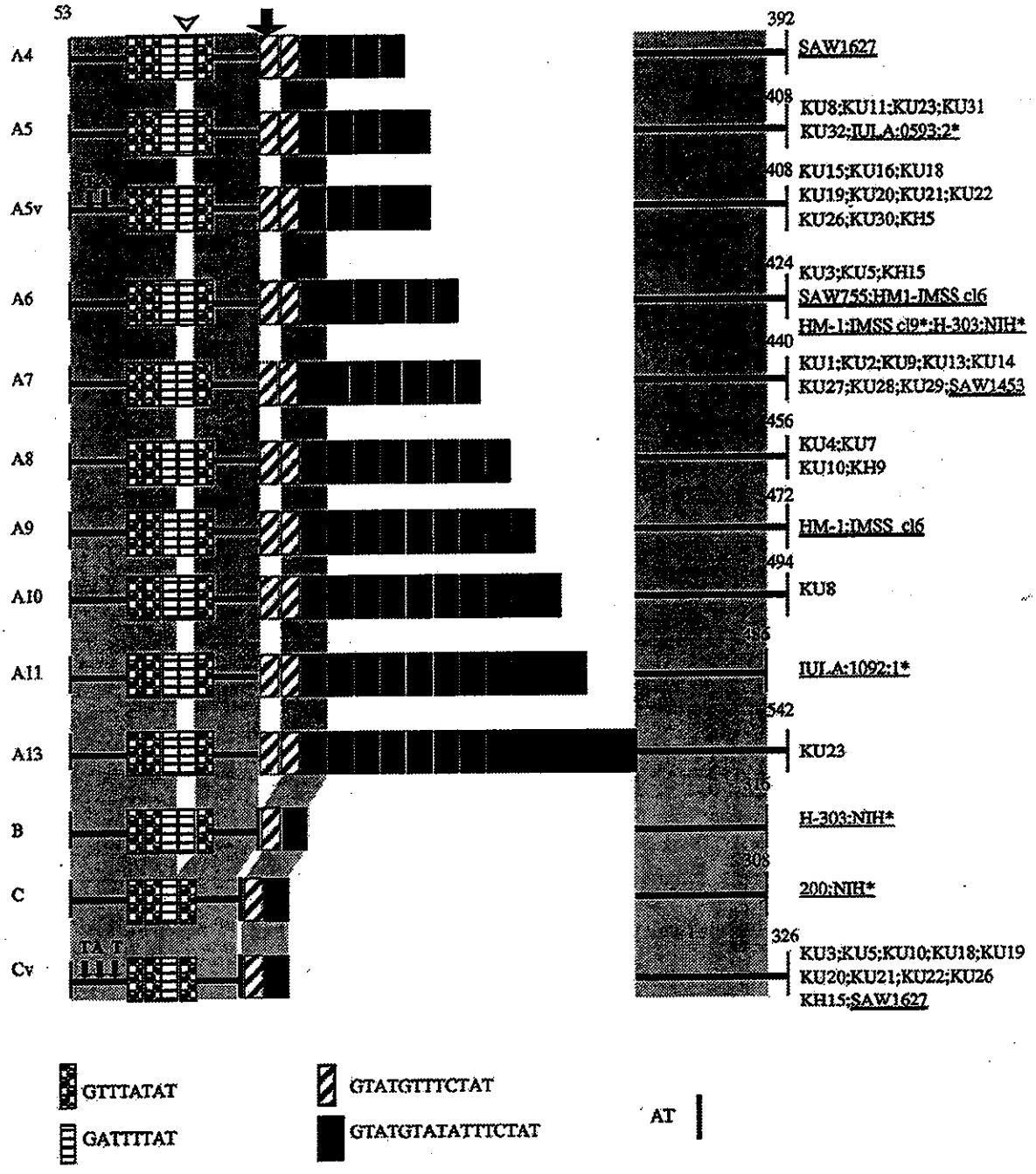

FIG. 3. Schematic representation of polymorphism in locus 5-6 among the Japanese isolates and reference strains. The numbers shown correspond to nucleotide numbers of locus 5-6 of HM-1:IMSS cl9 (GenBank/EMBL/DDBJ accession number AF276060). Sequences prior to nucleotide 52 are not shown for the reason described in the legend to Fig. 2. The first GTATGTTTCTAT and the second GATTTTAT repeats described in text are marked with a thick arrow and an arrowhead, respectively. Variant forms of A5 and C, in which two and three nucleotides were replaced (indicated by thin arrows), are designated A5v and Cv, respectively. An asterisk next to an isolate designation indicates that more information can be found in reference 34.

apparently single SREHP band had mixed nucleotide sequences (Table 1), likely due to a mixture in the apparently single PCR bands, which also indicates the heterozygosity of the SREHP locus. The hypothesis that some apparently single SREHP PCR fragments, e.g., HM1:IMSS cl6 (Fig. 1D), consist of two DNA sequences was also confirmed by sequencing individual SREHP fragments from HM-1:IMSS cl6 after cloning into a plasmid (data not shown). Three isolates that showed two SREHP bands that were different in size (KU9, KU11, and SAW755) revealed independent SREHP genotypes, i.e., G/M,

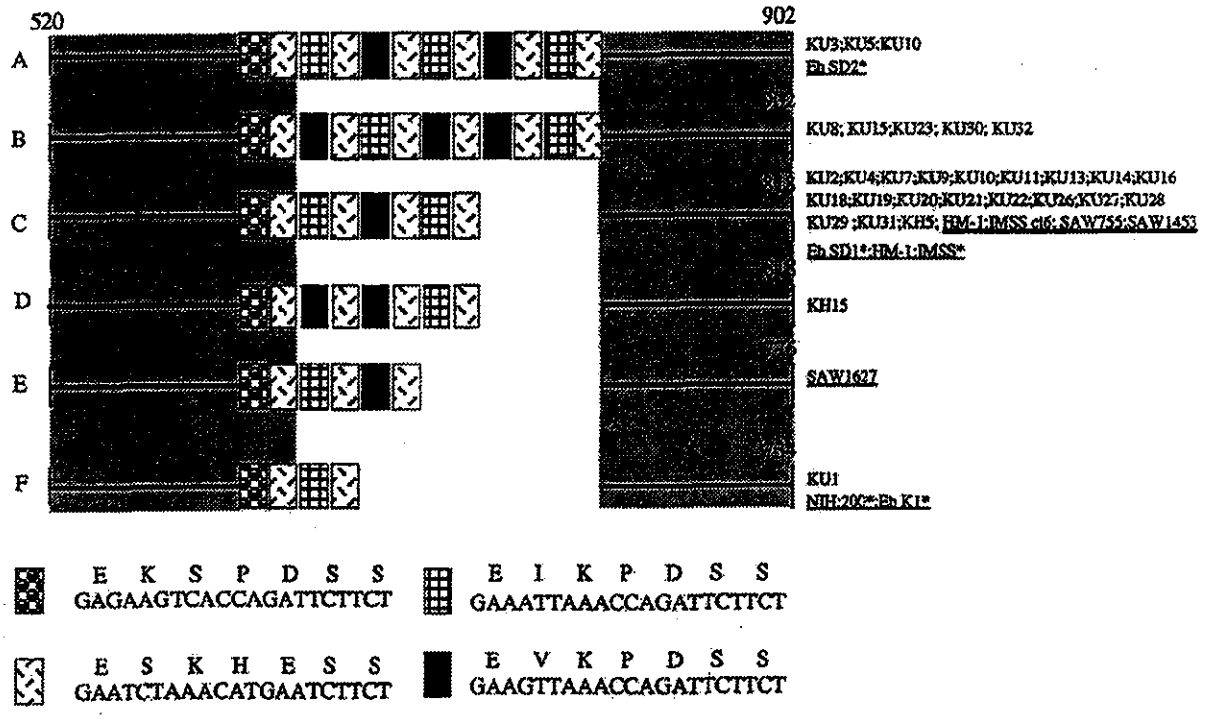

FIG. 4. Schematic representation of polymorphism in the repeat-containing region of the chitinase gene among the Japanese isolates and reference strains. The numbers shown in this figure correspond to nucleotide numbers of chitinase of HM-1:IMSS (GenBank/EMBL/DDBJ accession number U78319). Nucleotide and deduced amino acid sequences of heptapeptide repeats are also shown. An asterisk next to an isolate designation indicates that more information can be found in reference 12.

B/L, and C/N. In contrast to the previous studies (12), where SREHP fragments corresponding to types P and Q were amplified from HM1:IMSS, two fragments corresponding to types O and P (Fig. 5), which differ only in the presence or absence of a 18-bp repeat, were obtained.

Correlations between genotypes and origins of isolates. We investigated whether there is any correlation between the genotypes and the origins of the isolates (i.e., from male homosexuals or residents of institutions). The genotypes found in isolates from male homosexuals are highly polymorphic; all of these isolates are independent based on classification using these four loci (Table 1). In contrast, isolates obtained from residents of institutions showed less complex genetic polymorphisms. In addition, the genotypes of isolates obtained from mass infections at a single institution were indistinguishable (e.g., KU13 and -14, KU18 to -22, and KU27 to -29). Although the number of isolates tested was not large enough to enable statistical analysis, locus 1-2/types C and F; locus 5-6/type A5v/Cv; and SREHP/types A, H, and K were found exclusively in the isolates from institutions. In contrast, locus 1-2/types D, E, and G; locus 5-6/types A13/A5, A10/A5, A8, A8/Cv, A6/Cv, A5, and A5v; chitinase/types A, B, D, and F; and SREHP/types B/L, E, F, G/M, I, and M were never found in isolates from institutions but were found exclusively in isolates from male homosexuals. No apparent correlation was found either between genotypes and zymodemes or between genotypes and clinical manifestations (Table 1).

DISCUSSION

In the present study, we identified a large number of novel genotypes of four independent polymorphic loci among the Japanese isolates: 5 for locus 1-2, 6 for locus 5-6, 2 for chitinase, and 10 for SREHP. Combining these four independent polymorphic loci, all of the Japanese isolates were clearly distinct from any of the reference strains and also distinct from one another, except for the cases described below. Although genetic polymorphism among *E. histolytica* isolates from different geographic areas has been demonstrated (12, 34), the presence of extensive polymorphisms among the Japanese isolates in a limited geographic area and social populations, seen in the present study, reinforces a notion that genotyping of *E. histolytica* isolates by using these four polymorphic loci could serve as a tool to fingerprint individual isolates.

All 19 isolates obtained from male homosexuals were independent, which strongly indicates that the *E. histolytica* population in Japanese male homosexuals consists of a complex clonal structure. In contrast, *E. histolytica* strains from residents of institutions revealed a lower degree of polymorphism than those from male homosexuals. Considering the degree of polymorphism observed among the isolates derived from male homosexuals, it is surprising, but conceivable, that genotypes of isolates obtained from a single mass infection event in an institution for the mentally handicapped (e.g., isolates KU13 and -14, KU18 to -22, and KU27 to -29 [Table 1]) were identical. This fact suggests that a mass infection is likely caused by

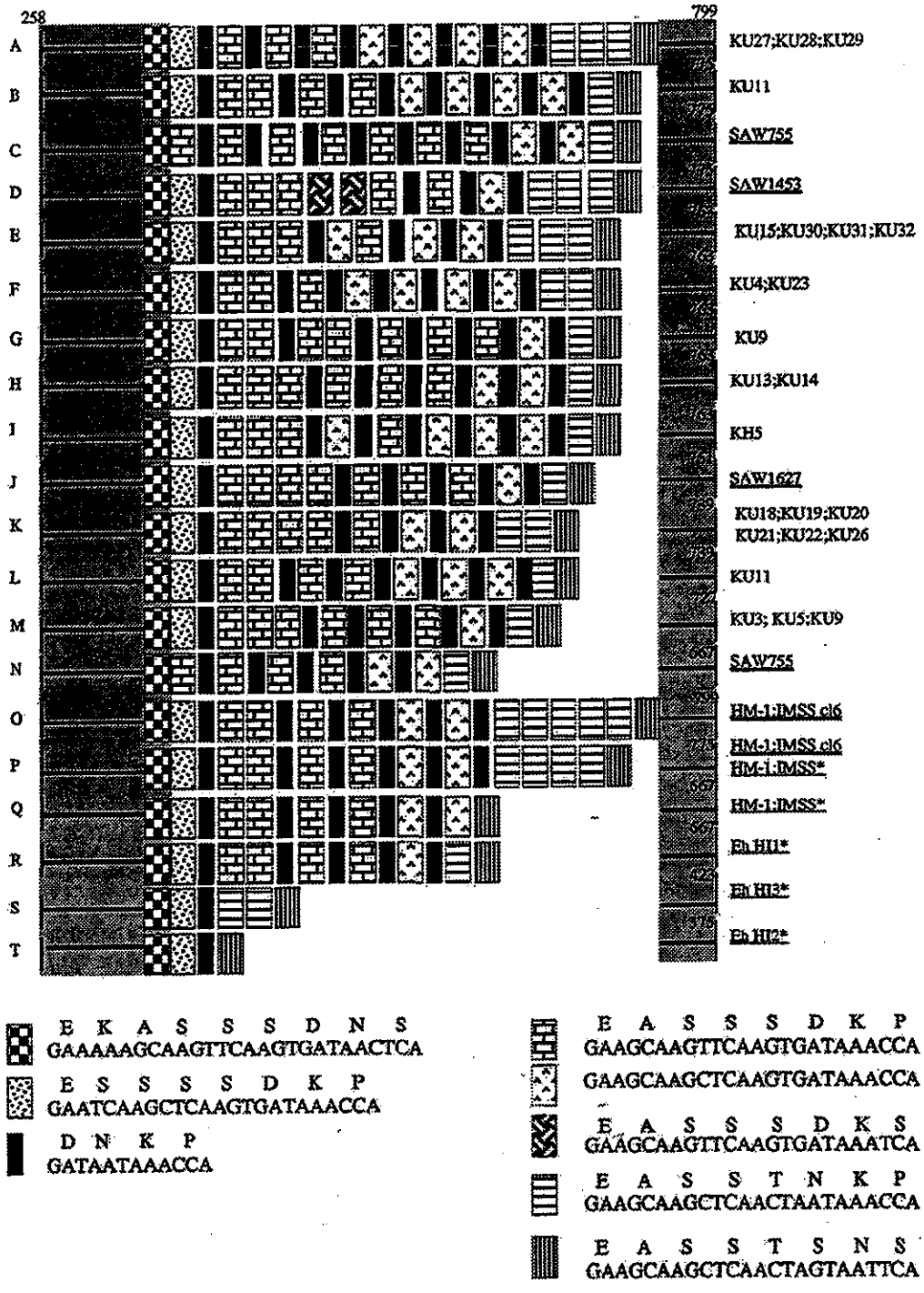

FIG. 5. Schematic representation of polymorphism in the repeat-containing region of the SREHP gene among the Japanese isolates and reference strains. The numbers shown in this figure correspond to nucleotide numbers of SREHP of HM-1:IMSS (GenBank/EMBL/DDBJ accession number M80910). Nucleotide and deduced amino acid sequences of tetra-, octa-, and nonapeptide repeats are also shown. An asterisk next to an isolate designation indicates that more information can be found in reference 12.

a single genotypic strain, and this is presumably due to introduction of a single *E. histolytica* strain into an institution. It is very striking that isolates from six mass infection cases in two independent events (KU18 to -22 and KU26 [Table 1]) that occurred 6 years apart (1994 and 2000) at remote geographic locations (data not shown) revealed an identical genotype.

DNA sequence polymorphisms of a limited number of axenized strains and stool samples have been described previ-

ously (12, 34). In the present study, we used a large number of xenic isolates because axenization may select certain genotypes. In addition, most of the strains were cryopreserved immediately after cultures were established and were revived only prior to the present study, as described in Materials and Methods, to minimize possible genotype changes. We also used uncultivated clinical specimens to avoid any selection during in vitro cultivation.

The genotypes of the widely used HM-1:IMSS are virtually indistinguishable in all of the loci tested except for the SREHP locus (7, 12, 34). The fact that the two SREHP sequences obtained from HM1:IMSS varied between Samuelson's and our laboratories, i.e., types O and P in this study and types P and Q previously (12), may indicate that PCR of this locus is prone to artifacts, as previously suggested (12). However, PCR amplification of the SREHP locus from this strain, previously shown by Clark and Diamond (7), apparently revealed no band corresponding to type Q but showed double bands that appeared to be consistent with types O and P in the present study. Although the reasons for this discrepancy are unknown, our demonstration that some of the apparently single PCR bands consist of at least two sequences argues against a proposal (12) that one out of two SREHP fragments was lost during PCR amplification. Thus, we concluded that the genotypes of the widely used reference strain are stable. A recent paper by Zaki et al. (35) also reported that genotypes are stable over time in culture and in the same patient. Homozygosity of the chitinase locus, tentatively deduced from a previous analysis of five isolates (12), was confirmed for all the isolates tested, with one exception (KU10). The presence of two chitinase bands in the KU10 isolate appears to be due to heterozygosity of this locus, rather than indicating the possibility of a mixed culture, for the following reasons. First, the KU10 strain has been xenically maintained for more than 10 years, during which time one strain likely would have outgrown the other if the culture was initiated as a mixture. Second, no mixed genotype of locus 1-2, which is homozygous in all of the isolates tested, was observed in KU10. However, the significance of heterozygosity at the chitinase locus in this isolate is unknown. The finding that the repeat-containing region of chitinase is the least polymorphic among the four loci, together with the fact that chitinase appears to be homozygous in most of our isolates, strongly indicates that there are functional constraints on chitinase polymorphism, which is also consistent with the hypothesis of a bottleneck spread of *E. histolytica* isolates, proposed by Ghosh et al. (12). Such functional constraints likely include the structural requirements of the enzyme for catalysis and/or multimerization.

The high genetic polymorphism among *E. histolytica* isolates described in this work, as with polymorphisms described for *Leishmania* and *Trypanosoma cruzi* (3, 23, 29), implies similarly diverse biological characteristics such as immunopathological effects, drug sensitivities, and vaccine attributes. The polymorphic nature of SREHP requires further attention because SREHP is being exploited as a protective immunodominant antigen (36, 37). Finally, we propose that molecular typing of ameba isolates by using these polymorphic loci should help in determining geographic origins of isolates and routes of transmission. Analysis of genotypes of *E. histolytica* isolates from a variety of geographic locations, e.g., Southeast Asia, is in progress.

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ORIGINAL PAPER

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Possible role of calcium ions, calcium channels and calmodulin in excystation and metacystic development of *Entamoeba invadens*

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Abstract The effect of calcium ions (Ca^{2+}) and calmodulin (CaM) on the excystation and metacystic development of *Entamoeba invadens* was examined by transfer of cysts to a growth medium containing calcium antagonists and CaM inhibitors. Excystation, which was assessed by counting the number of metacystic amoebae after induction of excystation, was inhibited by the calcium chelators ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetate (EGTA) and ethylene-diaminetetraacetate (EDTA), with EDTA being more potent than EGTA. The inhibitory effect of higher concentrations of these chelators on excystation was associated with reduced viability of cysts. Metacystic development, when determined by the number of nuclei in an amoeba, was delayed by EGTA, because the percentage of four-nucleate amoebae was higher than in controls at day 3 of incubation. EDTA made metacystic development unusual by producing a large number of metacystic amoebae with more than ten nuclei. The inhibition of excystation by these chelators was partially abrogated by their removal. A putative antagonist of intracellular calcium flux, 8-(*N*,*N*-diethylamino) octyl-3,4,5-trimethoxybenzoate (TMB-8) also inhibited the excystation and metacystic development, but had little effect on cyst viability. The slow Na^{+}-Ca^{2+} channel blocker bepridil but not verapamil inhibited the excystation and metacystic development, associating with reduced cyst viability at higher concentrations. The inhibitory effect of bepridil on excystation was abrogated by removal of the drug. The CaM inhibitor trifluoperazine (TFP) but not W-7 [*N*-(6-aminohexyl)-chloro-1-naphtalene sulphonamide] inhibited the excystation and metacystic development. The inhibitory effect of TFP on excystation was also abrogated by removal of the drug. These results indicate that extracellular calcium ions, amoebic intracellular calcium flux, calcium channels, and a CaM-dependent process contribute to the excystation and metacystic development of *E. invadens*.

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Introduction

The excystation and metacystic development of *Entamoeba* spp. are basic to the initiation of infection. Despite the importance of understanding these processes for the control of amoebiasis, studies on these processes are very limited. Although they have been described previously for *Entamoeba histolytica* (Dobell 1928; Cleveland and Sanders 1930), little is known about the mechanisms involved. Since *E. histolytica* cannot be induced to encyst in axenic culture, studies have been conducted on axenic in vitro encystation of *Entamoeba invadens*, a reptile parasite, as a useful model for encystation of *E. histolytica* (López-Romero and Villagómez-Castro 1993), because of its close similarity with *E. histolytica* in morphology and life cycle (McConnachie 1969). Since the excystation and metacystic development of *E. histolytica* (Dobell 1928; Cleveland and Sanders 1930) and of *E. invadens* (Geiman and Ratcliffe 1936) look similar, in vitro excystation of *E. invadens* may also become a useful model for excystation of the human parasite. Excystation of *E. invadens* can be induced in vitro by the transfer of cysts from an encystation medium to a growth medium (McConnachie 1955; Rengpien and Bailey 1975; Garcia-Zapien et al. 1995).

Calcium ions (Ca^{2+}) play a central role in various biological functions in eukaryotic cells. Ca^{2+}-dependent metabolic processes are often mediated by an intracellular calcium receptor, calmodulin (CaM), in cell proliferation and differentiation (Cheung 1980; Klee et al.

1980). Several types of Ca^{2+} and CaM antagonists, which interfere with Ca^{2+}-dependent metabolic processes, have been used to elucidate the role of Ca^{2+} and CaM in these processes (Cheung 1980; Klee et al. 1980). The in vitro cytolytic activity of *E. histolytica* was inhibited by the chelation of extracellular Ca^{2+} with ethyleneglycol bis (β-aminoethyl ether)-*N,N'*- tetraacetate (EGTA), by a putative antagonist of intracellular calcium flux, 8-(*N,N*-diethylamino) octyl-3,4,5-trimethoxybenzoate (TMB-8), and by the calcium channel blockers, bepridil and verapamil, indicating that Ca^{2+} plays an important role in the parasite-induced death of target cells (Ravdin et al. 1982, 1985). With regard to CaM, Muñoz et al. (1991) demonstrated that *E. histolytica* contained CaM, which functioned during the secretion of electron-dense granules containing collagenase. The participation of Ca^{2+} and CaM has been demonstrated in both the growth and encystation of *E. histolytica* and *E. invadens* (Makioka et al. 2001a). There are, however, no studies on the role of Ca^{2+} and CaM in the excystation and metacystic development of *Entamoeba*. We report here that Ca^{2+} and CaM contribute to the excystation and metacystic development of *E. invadens*.

Materials and methods

Trophozoites of *E. invadens* strain IP-1 were cultured in axenic medium BI-S-33 (Diamond et al. 1978) at 26°C. To obtain cysts, trophozoites (5×10^5 cells/ml) were transferred to an encystation medium called 47% LG (LG is BI without glucose; Sanchez et al. 1994). After 3 days, cells were harvested and treated with 0.05% sarkosyl (Sigma, St. Louis, Mo.) to destroy trophozoites (Sanchez et al. 1994). The remaining cysts were washed with phosphate-buffered saline (PBS), counted, and suspended in a growth medium. Viability of cysts was determined by trypan blue dye exclusion. The agents used in the present study and their initial solvents were as follows: Ca^{2+} chelators EGTA and EDTA (distilled water); the putative inhibitor of intracellular Ca^{2+} flux TMB-8 (Malagodi and Chiou 1974) (distilled water); Ca^{2+} channel blockers bepridil (Vogel et al. 1979) (95% ethanol) and verapamil (Shigenobu et al. 1974) (distilled water); CaM inhibitors trifluoperazine (TFP) (Levin and Weiss 1977) (dimethylsulfoxide; DMSO) and W-7 [*N*-(6-aminohexyl)-chloro-1-naphtalene sulphonamide] (Hidaka et al. 1981) (50% ethanol). All agents were purchased from Sigma. For experiments on the excystation and metacystic development of *E. invadens*, duplicate cultures of 5×10^5 cysts/ml were incubated with various concentrations of these agents for 3 days. Metacystic amoebae and cysts were counted in a hemocytometer and their viability determined by trypan blue dye exclusion. Viable metacystic amoebae and cysts were clearly distinguished as light yellow and light blue in colour, respectively. The former was also identified by positive motility. Control cultures received the same volume of each solvent.

Metacystic development was determined by the number of nuclei per amoeba. Cells were harvested at days 1 and 3 and stained with modified Kohn (Kumagai et al. 2001). The number of nuclei per amoeba was determined by double-counting at least 100 amoebae.

For experiments on the reversibility of the effect of agents, duplicate cultures containing each agent were incubated for 1 day. Cells were then centrifuged at 400 *g* for 5 min after chilling on ice and the spent medium removed. Cells were washed twice with a growth medium and then resuspended in a fresh growth medium. In control cultures, cells were similarly treated without replacement of the medium. The cultures were incubated for an additional 2 days, and metacystic amoebae were counted.

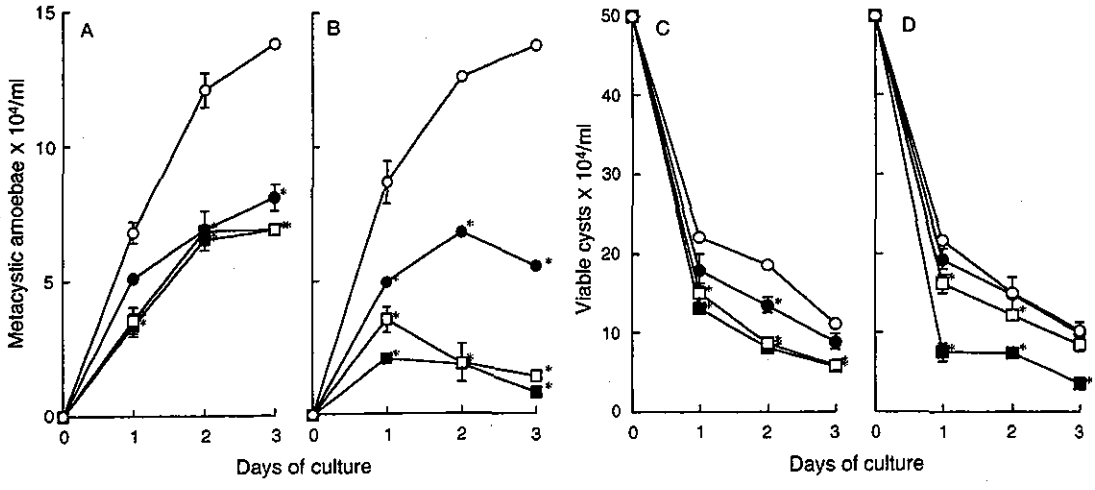
All experiments were performed at least three times and similar results were obtained in each replicate. Therefore, representative data from duplicate cultures are shown in the results.

Results

Effect of Ca^{2+} chelators on excystation and cyst viability

The number of metacystic amoebae at day 1 of incubation with 1 mM EGTA was hardly reduced compared to controls, whereas it was significantly reduced at 5 and 10 mM (Fig. 1A). Metacystic amoebae in control cultures increased sharply in number from day 1 to day 3. In contrast, a gradual increase in the number of metacystic amoebae occurred from day 1 to day 3 in cultures exposed to EGTA. The number of metacystic amoebae at day 1 with 1–10 mM of EDTA was also reduced

Fig. 1A–D Effect of EGTA and EDTA on the number of metacystic amoebae and cyst viability of *Entamoeba invadens*. Cysts were transferred to a growth medium containing various concentrations of EGTA (**A**, **C**) and EDTA (**B**, **D**). Mean number ± SE of metacystic amoebae (**A**, **B**) and viable cysts (**C**, **D**) for duplicate cultures are plotted (*$P < 0.05$). Concentrations shown by *open circles*, *solid circles*, *open squares*, and *solid squares*, are 0, 1, 5, and 10 mM, respectively



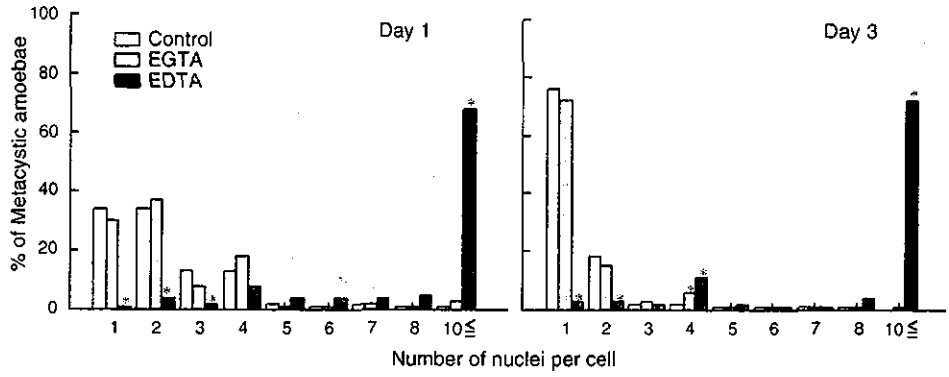


Fig. 2 Effect of EGTA and EDTA on metacystic development of *E. invadens*. Cysts were transferred to a growth medium with or without 10 mM EGTA or 5 mM EDTA. The nuclei per metacystic amoeba stained with modified Kohn at day 1 and 3 of incubation were counted and the percentage of amoebae was determined (*$P < 0.05$)

compared to controls and thereafter no increase in the number of amoebae occurred from day 1 to day 3 in cultures exposed to 5 and 10 mM (Fig. 1B).

As shown in Fig. 1C, the number of viable cysts at day 1 in cultures containing 5 and 10 mM of EGTA decreased by 32% and 41% of the control, respectively, whereas the number had decreased by 26% and 65% of the control, respectively, in cultures containing the same concentrations of EDTA (Fig. 1D).

Effect of Ca^{2+} chelators on metacystic development

As shown in Fig. 2, the percentage of metacystic amoebae with four nuclei at day 1 of incubation in cultures containing 10 mM EGTA was similar to that in controls, but at day 3 it was higher than in controls, suggesting an inhibitory effect of EGTA on metacystic development. In contrast, 68% and 72% of metacystic amoebae at days 1 and 3, respectively, in cultures containing 5 mM EDTA had more than ten nuclei.

Reversibility of effect of Ca^{2+} chelators on excystation

To determine whether the inhibitory effect of EGTA and EDTA on excystation was reversible, spent medium containing 10 mM EGTA or EDTA for 1 day of incubation was replaced with a drug-free growth medium. As shown in Fig. 3, the number of metacystic amoebae increased to 72% ($P < 0.05$) and 40% ($P < 0.05$) of control for EGTA and EDTA, respectively, after their removal.

Effect of TMB-8 on excystation and cyst viability

The number of metacystic amoebae at day 1 in cultures containing 500 μM TMB-8 was reduced compared to controls (Fig. 4A). Thereafter, amoebae increased in number slowly from day 1 to day 3 in cultures with 300 and 500 μM compared to controls (Fig. 4A). TMB-8 at 100–500 μM had no effect on cyst viability at day 1 and 2 (Fig. 4B). Metacystic development was delayed in

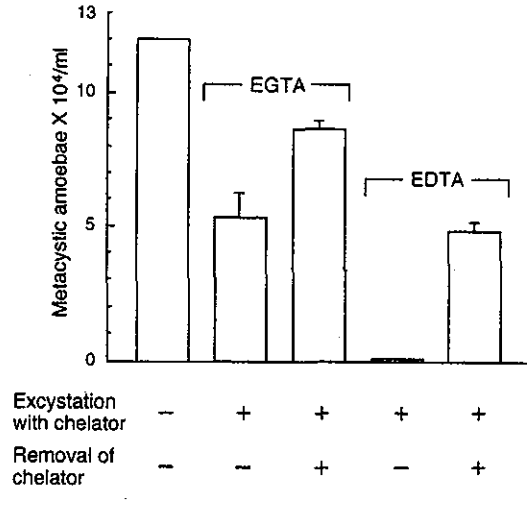


Fig. 3 Effect of removal of EGTA and EDTA on excystation of *E. invadens*. After exposure of cysts to 10 mM EGTA or EDTA in growth medium for 1 day, the chelators were removed by replacement of the medium with drug-free growth medium and the cultures were further incubated for 3 days. Mean number + SE of metacystic amoebae for duplicate cultures are plotted

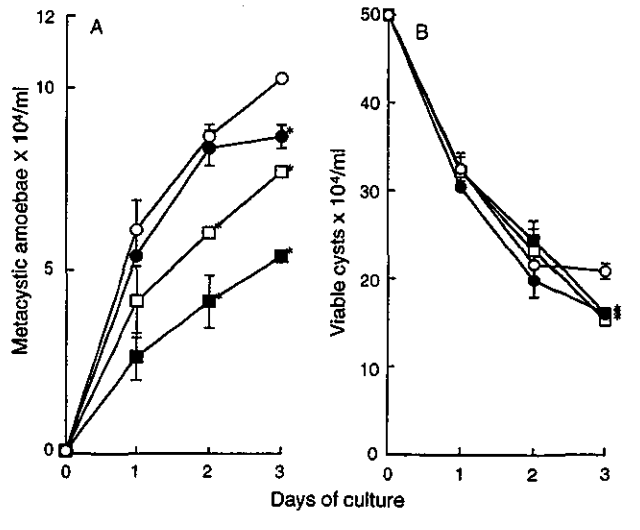


Fig. 4A, B Effect of TMB-8 on the number of metacystic amoebae and cyst viability of *E. invadens*. Mean number ± SE of metacystic amoebae (**A**) and viable cysts (**B**) for duplicate cultures are plotted (*$P < 0.05$). Concentrations shown by *open circles*, *solid circles*, *open squares*, and *solid squares*, are 0, 100, 200, and 500 μM, respectively

cultures containing 500 μM TMB-8 compared to controls (data not shown).

Effect of Ca^{2+} channel blockers on excystation and cyst viability

Bepridil at 50 and 100 μM reduced the number of metacystic amoebae at day 1 and 2 compared to controls (Fig. 5A). In contrast, verapamil at the same concentrations had no effect (Fig. 5B). Bepridil at 100 μM reduced the number of viable cysts during incubation (Fig. 5C), whereas verapamil had no effect (Fig. 5D).

Effect of bepridil on metacystic development

As shown in Fig. 6, 12% of metacystic amoebae were four-nucleate and 84% of those were one- to three-nucleate at day 1 of incubation in control cultures, whereas 47% of amoebae were four-nucleate and 49% of those were one- to three-nucleate in cultures containing 100 μM bepridil, suggesting the inhibition of metacystic development in the presence of bepridil. At day 3, only 1% of amoebae were four-nucleate and the percentage of one-nucleate amoebae reached 82% in controls, whereas 29% of amoebae were still four-nucleate and only 20% were one-nucleate in cultures with the drug.

Reversibility of effect of bepridil on excystation

To determine whether the inhibitory effect of bepridil on excystation was reversible, spent medium containing 100 μM bepridil for 1 day of incubation was replaced with drug-free growth medium. After removal of the drug, the number of metacystic amoebae increased to 82% ($P < 0.05$) of the control (Fig. 7).

Effect of CaM inhibitors on excystation and cyst viability

TFP at 50 and 100 μM reduced the number of metacystic amoebae at day 2 and 3 and during incubation, respectively, compared to controls (Fig. 8A), whereas W-7

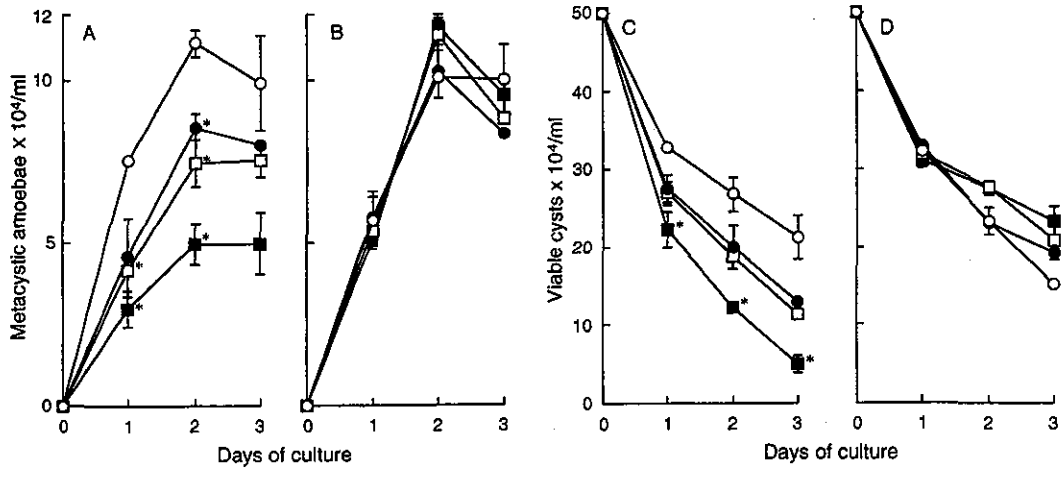


Fig. 5 Effect of bepridil (**A**, **C**) and verapamil (**B**, **D**) on the number of metacystic amoebae and cyst viability of *E. invadens*. Mean number ± SE of metacystic amoebae (**A**, **B**) and viable cysts (**C**, **D**) for duplicate cultures are plotted (*$P < 0.05$). Concentrations shown by *open circles*, *solid circles*, *open squares*, and *solid squares*, are 0, 10, 50, and 100 μM, respectively

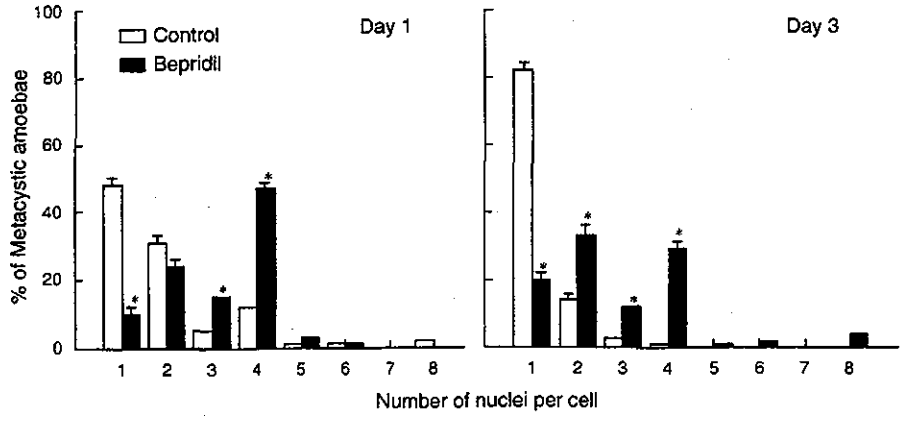


Fig. 6 Effect of bepridil on metacystic development of *E. invadens*. Cysts were transferred to a growth medium with or without 100 μM bepridil. The nuclei per metacystic amoeba stained with modified Kohn at day 1 and 3 of incubation were counted and the percentage of amoebae was determined (*$P < 0.05$)